







PHYSICAL  
BIOCHEMISTRY





# PHYSICAL BIOCHEMISTRY

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SECOND EDITION

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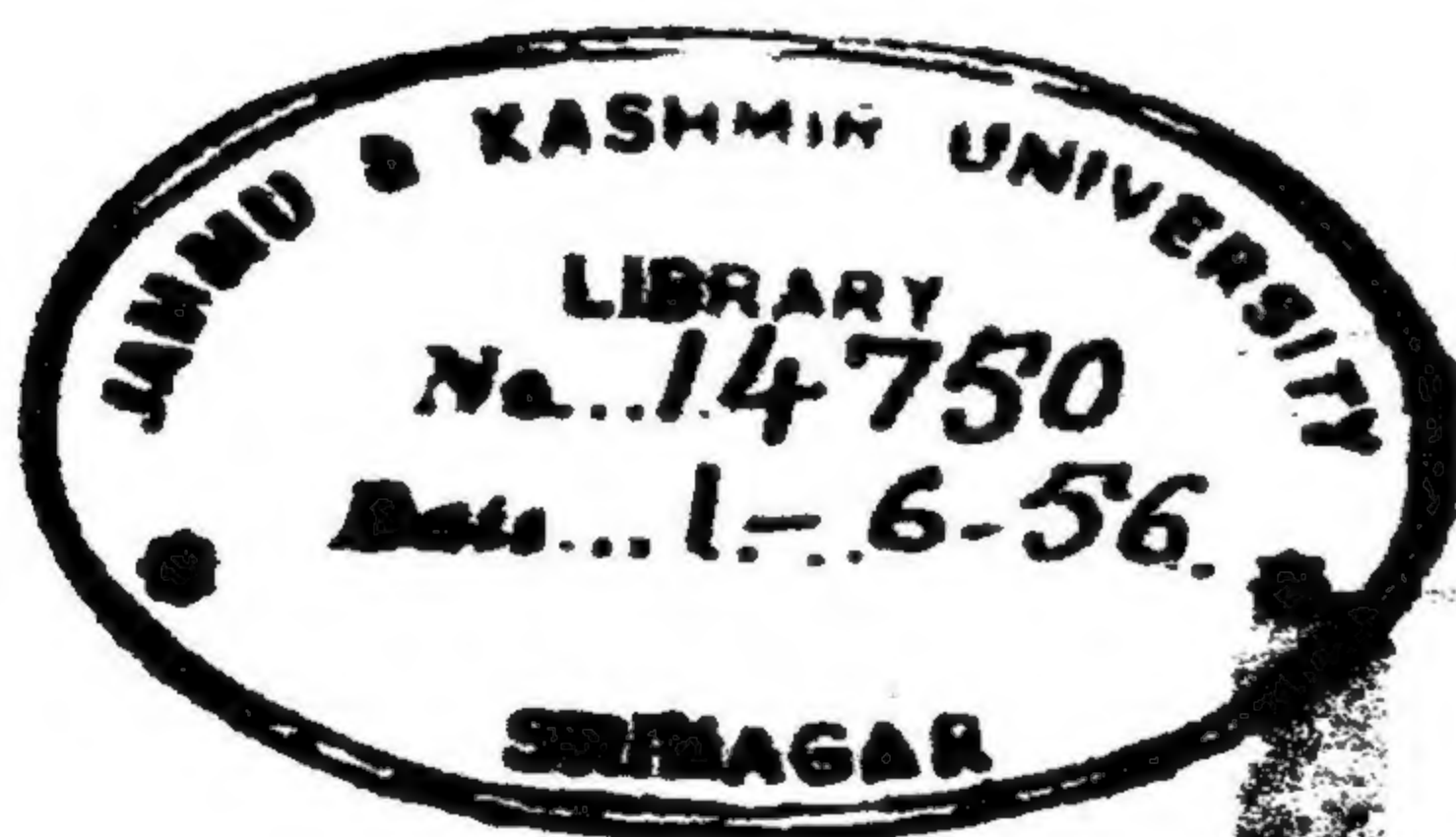
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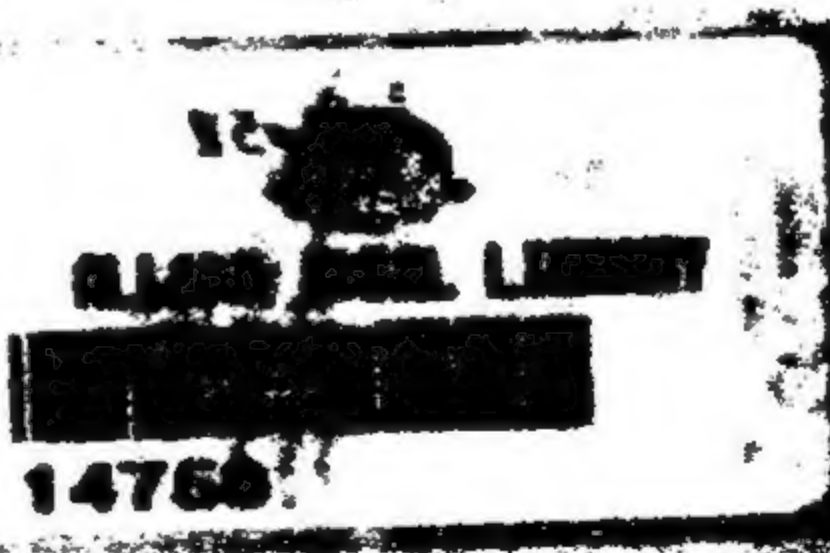
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## PREFACE TO THE SECOND EDITION

The purpose of this edition, as was that of the first, is to bring to the student of the biological sciences a picture of physical chemistry which is meaningful to him. An experimental rather than a theoretical approach has been adopted.

One is constantly impressed by the fragmentary character of contemporary science. It is imperative that our graduate students acquire a broad training in the basic sciences so that they can develop a background which will enable them to integrate to some extent the enormous and diverse material with which they are faced. I believe a knowledge of physical chemistry is of greater assistance in this respect than that of any other basic science.

The revision has involved considerably more labor than was anticipated; it was necessary to limit the size of the book, severely and this limitation has meant a rather ruthless exclusion of much which I should have liked to include and very careful editing of that which remains. It would have been easier to write a more lengthy volume. It is hoped, however, that the book has gained in compactness and unity. About two-thirds of the first edition has been rewritten and new material woven into the pattern of the text.

I am grateful for the very industrious help of my graduate student Victor H. Baptist in checking the problems and for his zealous reading of proofs.

HENRY B. BULL

*Chicago, Illinois*

*May 1, 1957*





## PREFACE TO THE FIRST EDITION

G. N. Lewis once defined physical chemistry as a science which includes everything which is interesting and excludes everything which is uninteresting. Evidently, the subject of physical chemistry is a very broad one, and invariably a considerable selection of topics to be presented must be made. I have tried to choose material which aids in the interpretation of living systems. It has long been my feeling that instruction in physical chemistry for pre-medical and for graduate students in the biological sciences is not as well adjusted to the needs of such students as it could be. This book is an attempt to reorient to some extent this type of instruction.

The volume is an outgrowth of a series of about thirty-six lectures which I have given in alternate years to graduate students in biochemistry, physiology, bacteriology, and neurology. The lectures were also attended by medical students. The time is short; the material could be treated more conveniently in about forty-eight lectures. As it is essentially an outline course and must be supplemented by a generous amount of outside reading, literature citations are included for this purpose. To the more serious student of physical chemistry I recommend *Text Book of Physical Chemistry* by S. Glasstone, published by D. Van Nostrand Company, New York City.

I make no claim for completeness in my presentation of physical biochemistry. Much work has been omitted, not because I did not think it sound or important, but because it did not seem to fit into the story I was telling.

I wish to thank Doctors D. R. Briggs, M. Dole, E. Guth, R. S. Livingston, H. Neurath, and K. Sollner for their critical comments on the sections of the manuscript they were asked to read. They saved me from many a pitfall and I am duly grateful. They are not, however, to be blamed for any errors which remain. For these and for the method of presentation I alone am responsible.

This book was tested for clarity on John A. Cooper, who served as a highly intelligent guinea pig. My only concern is that he may have misled me because what was clear to him might be obscure to a less talented graduate student. Martin Gutmann also contributed suggestions for the betterment of the manuscript. I am grateful to both these students for their help.

Ross Aiken Gortner, to whom I am most indebted, never saw the manuscript or the book. It is to him, however, that I owe my interest and a large part of my knowledge of physical chemistry. His recent death was a great

## PREFACE TO THE FIRST EDITION

My dear friends, those who knew him. I am proud to be, in a manner of speaking, his official successor.

This book is dedicated to Fredrica Jane and Fredrica Jean.

HENRY B. BULL

London, 1943  
New York, 1943

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## ATOMS and MOLECULES

Atoms and molecules are the building blocks from which the chemist makes his science, and it is necessary that we have clearly in mind some of their more important characteristics.

Matter is practically transparent when bombarded with elementary particles, indicating a great deal of open space in atoms. Indeed, nuclear radii are of the order of  $10^{-12}$  to  $10^{-13}$  centimeter; the atomic radii are of the order of  $10^{-8}$  centimeter. The electrons are situated around the nucleus and carry a negative charge. Their mass is small and their radii are approximately  $3 \times 10^{-13}$  centimeter. The nucleus is made up of protons and neutrons. Protons have a unit positive charge, and their masses are 1836 times those of the electrons. Neutrons have masses very nearly equal to those of protons, but the net charge on neutrons is zero. When a proton is converted into a neutron a positive electron or positron is emitted.

The hydrogen atom has one electron rotating about a proton. This is the simplest atom. The next atom as we ascend the scale of complexity is the helium atom with two protons and two neutrons in the nucleus about which rotate two electrons. Successive atoms are constructed by adding planetary electrons and by increasing the charge in the nucleus by the addition of protons; the number of protons in the nucleus of a neutral atom equals the number of planetary electrons. Neutrons must also be added to the nucleus to have a stable atom. Isotopes, which we shall discuss presently, result from the addition or subtraction of neutrons from the nucleus.

In the early atomic picture, the electrons were supposed to rotate around the nucleus as the planets rotate about the sun. This conception had to be abandoned for two reasons. 1. Such rotating electrons would be constantly accelerated and, accordingly, would lose energy by emission and would eventually fall in on the nucleus. 2. Such electrons would contribute to the specific heats of the system; it is known that they do not.

These considerations led to the formulation of the quantum theory of the atom. It is postulated that an atom exists in stable states with definite energies. Transition between these states leads to the emission or absorp-

tion of light, the frequency of which is directly related to the difference in energy between the two states. That is,  $E_1 - E_2$  equals  $h\nu$  where  $E_1$  and  $E_2$  are the energies in ergs,  $h$  is Planck's constant ( $6.54 \times 10^{-27}$  erg-second), and  $\nu$  is the frequency expressed in reciprocal seconds. A positive value of  $\nu$  corresponds to absorption, whereas a negative value corresponds to emission of radiation. The quantity  $h\nu$  is a quantum of energy.

Electrons can behave as waves or as particles. The quantum theory permits the dual behavior of the electrons to be reconciled. This wave-particle duality applies to the other elementary particles such as protons and neutrons as well as to electrons. A beam of such particles can be diffracted just as light can be diffracted; however, the velocity of the material particle can vary from zero to that of light itself. The wavelength of the beam of material particles is, for velocities very much less than the velocity of light,  $h/mu$ , where  $m$  is the mass of the particle and  $u$  is its velocity.

The electrons are arranged in shells. The first shell is called the K-shell, the second the L-shell, the third the M-shell, and so on out. These electrons describe wave functions around the nucleus, and the original picture of the planetary electrons is replaced by a more uniform structure in space, which can be considered as filling the whole atomic volume in spite of the fact that the electrons, when considered as particles, are small compared with the atom.

Each shell has in it one or more orbits which are occupied by electrons. No more than two electrons can occupy a single orbit, and, furthermore, these electrons must spin in opposite sense. Chemical reactions result when the wave functions of the outer electrons of two atoms overlap and lead to a decrease in energy. Molecular structure will be discussed presently.

From the electrons arise the emission spectra of the atoms. As is well known, if the temperature of an element is increased sufficiently, light will be emitted from the element; the wavelength of the light will be highly characteristic of the element and is utilized in spectroscopic analysis. This type of emission originates from the outer electrons which absorb energy and emit it in the form of light of definite wavelength. If the elements are bombarded with high-speed electrons, the electrons in the innermost shells become excited and, when they yield up their energy, the wavelength of the emitted radiation is very short. Such emitted rays are X-rays. X-rays from the K-shell have high energy and high penetrating power and are known as hard X-rays. The energy and penetrating power of the X-rays decrease with increasing distance from the nucleus.

In total, there are over 92 elements, but living systems make use of only about 20 of these. It is possible to arrange the elements in the form of a periodic table which exhibits the interrelationships familiar in inorganic chemistry. Shown in Table 1 is what one might call a biological periodic table which includes only those elements occurring naturally in living tissue.

# ATOMS AND MOLECULES

TABLE 1  
BIOLOGICAL PERIODIC TABLE

Group	O	I	II	III	IV	V	VI	VII	VIII
Sub-Group		A	B	A	B	A	B	A	B
Period									
1		${}^1\text{H}^1$							
2									
3		${}^{11}\text{Na}^{23}$	${}^{12}\text{Mg}^{24}$			${}^7\text{N}^{14}$	${}^8\text{O}^{16}$	${}^9\text{F}^{19}$	
4		${}^{19}\text{K}^{39}$	${}^{20}\text{Ca}^{40}$		${}^6\text{C}^{12}$	${}^{13}\text{Al}^{27}$	${}^{16}\text{S}^{32}$	${}^{17}\text{Cl}^{35}$	${}^{26}\text{Fe}^{56}$ ${}^{27}\text{Co}^{59}$
5		${}^{29}\text{Cu}^{63}$	${}^{30}\text{Zn}^{64}$			${}^{23}\text{V}^{51}$		${}^{25}\text{Mn}^{55}$ ${}^{33}\text{P}^{31}$	${}^{51}\text{I}^{127}$





# ARTIFICIAL ISOTOPES

3

TABLE 3

ABUNDANCE OF SOME NATURALLY OCCURRING ISOTOPES

Element	Atomic Number	Mass Number	Relative Abundance
Hydrogen	1	1	99.99
Deuterium	1	2	0.003
Carbon	6	12	99.3
Carbon	6	13	0.7
Nitrogen	7	14	99.86
Nitrogen	7	15	0.14
Oxygen	8	16	99.81
Oxygen	8	18	0.16

These ions are deflected by means of electrostatic and magnetic fields, the extent of deflection being an inverse measure of the mass of the atoms, whereas the intensity of the lines on a photographic plate is a measure of the amount of the element present.

## ARTIFICIAL ISOTOPES

If atoms of many elements are bombarded with high-speed particles ( $\alpha$ -particles, neutrons, protons, or deuterons) the elements will undergo nucleo reactions. The high-speed particles are derived from cyclotrons or from uranium piles, the details of which need not concern us here. The nucleo reactions are clearly transmutations of the elements. Ordinarily, however, the extent of such reactions is extremely limited and only small amounts of the new elements are formed.

Nucleo reactions can produce either stable or unstable isotopes. The unstable isotopes spontaneously disintegrate giving rise to a new element together with elementary particles. For example, if boron is bombarded with high-speed deuterons, it forms an unstable isotope of carbon



The carbon isotope then disintegrates to yield boron 11 and a beta particle. A beta particle is an electron which may carry a positive or a negative charge.



Frequently, when an element disintegrates, the excess energy appears as  $\gamma$ -rays.  $\gamma$ -rays are high-energy electromagnetic radiation of the same wavelength and character as X-rays.

The rate at which an unstable isotope disintegrates is a function of the constitution of the nucleus and cannot be altered by ordinary physical or

chemical means. The rate of decay is simply proportional to the amount of isotope present or

$$\frac{dn}{dt} = -\lambda n \quad (1)$$

where  $n$  is the number of radioactive atoms present,  $t$  is the time, and  $\lambda$  the disintegration constant. Integrating equation 1 and converting logarithms to the base 10 gives

$$\log \frac{n}{n_0} = -\frac{\lambda t}{2.3} \quad (2)$$

The half-life of an isotope is the time required for half of the radioactive isotope to disintegrate or the time at which  $2n$  is equal to  $n_0$ ; then

$$t_{1/2} = \frac{2.3}{\lambda} \log 2 = \frac{0.693}{\lambda} \quad (3)$$

Table 4 gives the half-life and the type of radiation of some isotopes that have been used in biological investigation.

TABLE 4

## PROPERTIES OF SOME RADIOACTIVE ISOTOPES

Isotope	Half-Life	Type of Radiation
${}^3_1\text{H}$	31 years	$\beta^-$
${}^{11}_6\text{C}$	20.35 minutes	$\beta^+$
${}^{14}_6\text{C}$	$1 \times 10^4$ years	$\beta^-$
${}^{24}_{11}\text{Na}$	14.8 hours	$\beta^-, \gamma$
${}^{32}_{15}\text{P}$	14.3 days	$\beta^-$
${}^{35}_{16}\text{S}$	87.1 days	$\beta^-$
${}^{38}_{17}\text{Cl}$	37 minutes	$\beta^-, \gamma$
${}^{42}_{19}\text{K}$	12.4 hours	$\beta^-$
${}^{45}_{20}\text{Ca}$	180 days	$\beta^-, \gamma$
${}^{59}_{26}\text{Fe}$	47 days	$\beta^-, \gamma$
${}^{131}_{53}\text{I}$	8 days	$\beta^-, \gamma$

The number of disintegrations in a given time may be estimated by means of a Geiger-Mueller counter. The Geiger-Mueller counter consists of a metal cylinder filled with an easily ionizable gas and containing an axial wire charged to a potential of several hundred volts. Upon disintegration, the radioactive isotope gives off charged particles which ionize the gas in the Geiger-Mueller counter. The gas is thus rendered conducting and there is a surge of current from the axial wire to the metal cylinder which is amplified sufficiently to operate a mechanical counter. Each charged particle entering the gas chamber is, accordingly, counted. Only a fraction

the total number of charged particles emitted actually enter the gas chamber; the actual fraction depends on the geometrical correction of the instrument.

### UNITS AND DEFINITIONS

A certain number of definitions and units used in tracer work are helpful to know.

An electron volt is the amount of energy acquired by an electron moving under a fall of potential of 1 volt and is equal to  $1.60 \times 10^{-12}$  erg. This unit is so small that ordinarily one deals with a million times this unit or 1 million electron volts, abbreviated 1 m.e.v.

The curie is the unit of comparison of the quantity of radioactivity. It is the amount of radioactive substance giving the same number of disintegrations as 1 gram of radium, namely,  $3.7 \times 10^{10}$  per second. This unit is rather large, and ordinarily the millicurie (1 mc. =  $3.7 \times 10^7$  disintegrations per second) or the microcurie (1  $\mu$ c. =  $3.7 \times 10^4$  disintegrations per second) are closer to experimental amounts.

The unit of radiation is the roentgen (r.) which is measured by the amount of ionization produced by X-rays absorbed by a given amount of air. The radiation dosage to be delivered to biological material is ordinarily specified in roentgens. Thus a dose of 500 r. delivered at one sitting to a local skin area is capable of producing reddening in 80 per cent of patients within 4 weeks of treatment.

### USE OF TRACERS IN BIOCHEMISTRY

Once the concentration of the isotope is determined by the appropriate method, the kind of information which can be obtained with stable isotopes is identical with that which can be obtained with radioactive isotopes. Since the isotopes of a given element have very nearly identical chemical properties, the living organism is unable to distinguish between molecules containing different isotopes of the same element. Deuterium can be an exception to this rule because its mass is twice as great as that of hydrogen and compounds containing deuterium or hydrogen can differ appreciably in properties (see Table 2). Deuterium in higher concentrations inhibits or poisons respiration and fermentation, and the general practice in studies or intermediary metabolism is to avoid deuterium concentrations higher than about 1 part of deuterium to 5 parts of hydrogen.

Intermediates in physiological reactions are identified by feeding a compound appropriately tagged with an isotope. Compounds related to that fed are isolated, and their isotopic content determined. If these chemically related compounds contain the isotope in excess of that naturally present, then these compounds must have been derived from the compound



ified. In using an isotope for tracing the fate of a compound in the body, care must be taken to place the atom in a position in the compound where it will not be readily exchanged for an isotopic atom incidentally present in the medium. Deuterium atoms in carboxyl, hydroxyl, amino, and other polar groups are extremely labile and exchange with the hydrogen of the aqueous medium very quickly. Deuterium of methyl and methylene groups is, in general, stable and is not removable by treating the compounds even at high temperatures with acid or with alkali. In physiological work with deuterium the tissues or compounds isolated from the tissues are burned to carbon dioxide and water. The deuterium content of the resulting water is measured by refractometric or density methods.

### ISOTOPE DILUTION

This method of employing isotopes has been extensively used for the assay of biological materials. If it is wished to measure the amount of a compound such as an amino acid in a mixture, the amino acid in question is tagged with the tracer and added to the unknown mixture. Any of this particular amino acid present in the mixture will act as an unlabeled diluent for the tagged amino acid. To determine the amount of the dilution and, accordingly, the amount of the amino acid in the mixture, it is only necessary to recover a portion of the added amino acid in the pure state instead of having to insist on a complete recovery.

When radioactive isotopes are used, the calculation of the extent of dilution by the carrier is quite simple because there is none of the radioactive tracer in the natural product to start with and the small change in molecular weight of the compound due to the difference in atomic weight of the isotopes can be neglected. Suppose  $x$  grams of the tagged compound with  $n_1$  counts per minute are added to a mixture containing  $y$  grams of the untagged molecule. The specific activity becomes  $n_2/(x + y)$ . The initial specific activity is  $n_1/x$ . Measuring the specific activity before and after dilution permits calculating the amount of  $y$  since  $n_1/x$  equals  $n_2/(x + y)$ .

In discussing isotopic concentrations, the term atom per cent excess is used, by which is meant the percentage of the isotope in excess of that normally present. To calculate the general case where a weight of carrier  $x$  of a molecular weight  $M_1$ , containing the atom per cent excess  $C_1$ , is added to  $y$  grams of normal isotopic concentration where molecular weight is  $M_2$ , we have

$$y = \left[ \frac{C_1}{C_f} - 1 \right] \frac{M_2}{M_1} x \quad (4)$$

where  $C_f$  is the atom per cent excess in the final mixture.<sup>1</sup>

<sup>1</sup> H. Gest, M. D. Kamen, and J. R. Reiner, *Arch. Biochem.* 12, 273 (1947).

Books that are especially useful for the study of atomic structure and radioactivity are: *The Structure of Matter* by F. O. Rice and E. Teller, John Wiley & Sons, New York, 1949; *Nuclear Radiation in Physics*, R. E. Lapp and H. L. Andrews, Prentice-Hall, New York, 1948; *Radioactive Tracers in Biology*, M. D. Kamen, Academic Press, New York, 1948; *Radioactive Indicators*, G. Hevesy, Interscience Publishers, New York, 1948.

### MOLECULES

As two atoms approach each other there is a weak attraction which is primarily electrostatic in nature. This is known as the van der Waals attraction force and varies inversely as the sixth power of the distance between the atoms. The energy of such a "bond" is about 1,000 calories per mole and is of importance in maintaining the crystal structure of many compounds. At shorter distances of separation a powerful van der Waals repulsive force becomes effective which is similar in nature and magnitude to the forces of chemical bond formation and is due to the overlapping of the electronic distribution of the respective atoms. In a crystal these van der Waals attractive and repulsive forces are balanced at a certain distance of approach of the atoms. By means of X-ray diffraction studies, it is possible to measure the distance separating atoms in a crystal and, accordingly, to assign a so-called van der Waals radius to the atom. This distance represents the closest approach that can be made to an atom without chemical bond formation. These radii are useful in constructing molecular models. Table 5 shows van der Waals radii of a few atoms.

TABLE 5

VAN DER WAALS AND COVALENT RADII OF SOME ATOMS IN Å

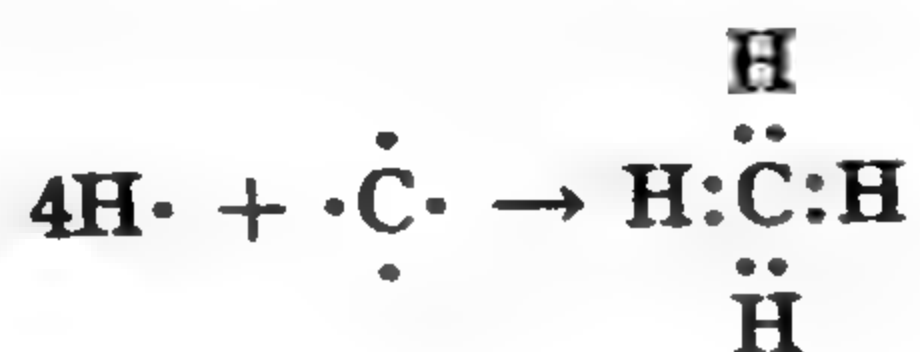
Atom	van der Waals	Covalent	
		Single Bond	Double Bond
H	1.2	0.30	
C	1.6	0.77	0.67
N	1.5	0.70	0.61
O	1.40	0.66	0.57
F	1.35	0.64	0.55
P	1.9	1.10	1.00
S	1.85	1.04	0.95
Cl	1.80	0.99	
Br	1.95		
I	2.15		

A chemical bond results when the repulsive forces are overcome and two atoms approach closer than the sum of their van der Waals radii. There are several forms which this interaction between the two atoms can assume

and which leads to the formation of different types of chemical bonds. The ionic bond involves a discrete transfer of electrons from one atom to the other, whereas a covalent bond results when two electrons are shared by the two atoms.

When the two interacting atoms differ greatly in their affinity for electrons, one of the atoms with the greater electron affinity will rob the other atom of an electron, thus leading to the creation of an ion pair. For example, sodium with one electron in its outer shell yields an electron to chlorine with 7 electrons in its outer shell. The sodium thus becomes positive while the chlorine becomes negative. In gaseous sodium chloride, the molecule exists as  $\text{Na}^+\text{Cl}^-$  and the bond between these atoms is an example of an ionic bond. When dissolved in water, the sodium and chloride become hydrated, leading to a separation into ions.

A covalent bond results when the two atoms do not differ greatly in their affinity for electrons. In such a bond there is a sharing of the electrons between the two atoms and these two electrons have opposite angular spins. For example, in the formation of methane from hydrogen and carbon we have

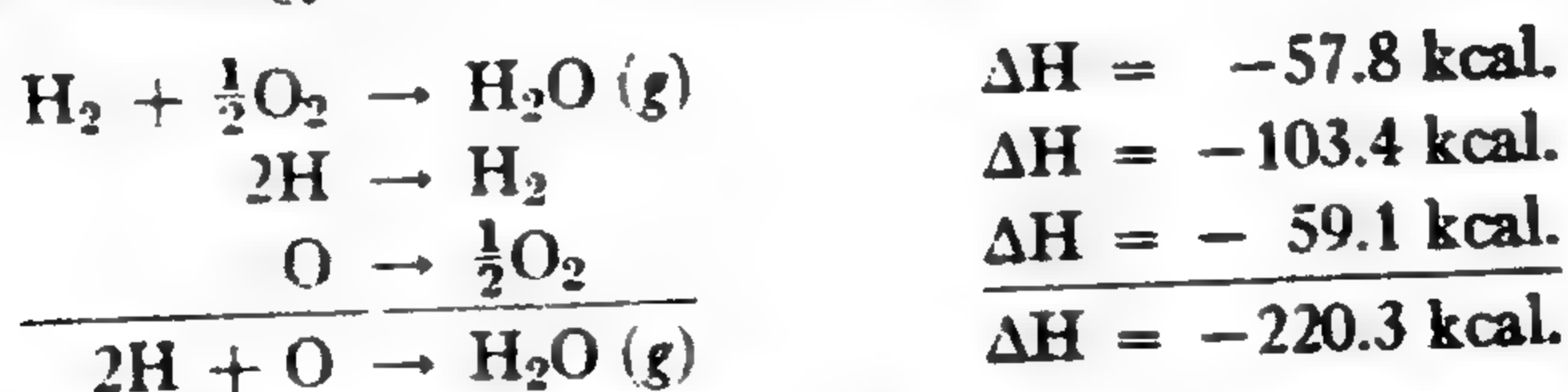


where the dots represent electrons; this is an example of an apolar bond. The dative or semipolar bond results when the shared electrons are supplied entirely by one atom.

### BOND ENERGIES

The term bond energy is defined as the energy that must be absorbed at 18° C. and at atmospheric pressure to break a bond of a gaseous molecule with the production of neutral gaseous atoms or radicals. The bond energy as defined does not represent strictly the internal energy of a bond; it represents rather the heat content of the bond. The energy of the C—H bond is, for example, one-fourth of the energy liberated in the reaction  $\text{C}(\text{g}) + 4\text{H}(\text{g}) \rightarrow \text{CH}_4(\text{g})$  where (g) represents the gaseous state.

Consider the energy of the O—H bond in water.



Rather arbitrarily the energy of each OH bond is found by dividing 220.3 by 2 or the energy for O—H bond is 110.2.



The calculation of bond energies is based upon the idea that bond energies are strictly additive, i.e., the sum of all the bond energies is equal to heat of formation of the molecule from the gaseous elements. Table 6 shows the bond energies of some chemical bonds.

TABLE 6

BOND ENERGIES IN KILOGRAM CALORIES

Bond	Energy	Bond	Energy
C—C	58.6	N—H	83.7
C—H	87.3	C=C	100
O—H	110.2	C=O	152
C—N	48.6	C=N	94
C—O	70.0	H—H	103.4
N—N	20		

## BOND DISTANCES AND ANGLES

The distance between atoms joined by valence bonds can be determined by spectroscopic methods, by X-ray crystal methods, and by electron diffraction of the gaseous molecules. The first method is limited to quite simple molecules. The second and third methods require that the molecular structure of the substance be known. It is found that the bond distances are quite constant from compound to compound provided that the situation is not complicated by resonance of which we shall speak presently. Furthermore, it has been found that the length of a bond between two atoms is the sum of the atomic radii. The radii of some atoms are shown in Table 5.

The angles formed by two covalent bonds of an atom can be determined from X-ray diffraction studies. These bond angles vary somewhat from compound to compound, but on the whole they show considerable uniformity. Table 7 gives a list of some bond angles.

TABLE 7

BOND ANGLES FOUND IN SEVERAL COMPOUNDS

Compound	Group	Bond Angle
H <sub>2</sub> O	H—O—H	105°
H <sub>2</sub> S	H—S—H	92° 20'
NH <sub>3</sub>	H—N—H	108°
N(CH <sub>3</sub> ) <sub>3</sub>	C—N—C	108°
Propane	C—C—C	111° 30'

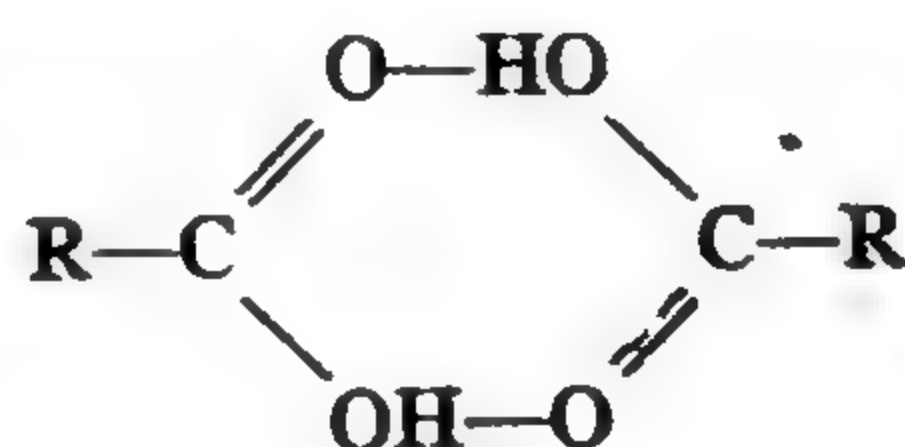
## HYDROGEN BONDS

One of the most interesting of chemical bonds as far as the biochemist is concerned is the hydrogen bond. The significance of this bond arises not

from its strength but from its weakness. Its usual strength is only in the neighborhood of 5 kilocalories. With such a low energy value it is easily possible for this bond to form and to rupture at normal temperatures.

The hydrogen bond is a chemical bond resulting from the attraction of two electronegative atoms for a proton. Accordingly, only the most electronegative atoms are capable of forming hydrogen bonds, and, furthermore, the strength of the bond increases with the electronegativity of the two bonded atoms. It is found that only fluorine, oxygen, nitrogen, and chlorine can form hydrogen bonds, and that the ability to form hydrogen bonds decreases in the above order.

There are numerous examples of hydrogen bonds or hydrogen bridges, as they are sometimes called. The fatty acids have a great tendency to form dimolecules. This structure involves two hydrogen bonds.



The hydrogen bond determines the magnitude and nature of the mutual interaction of water molecules and is consequently responsible for the striking physical properties of this uniquely important substance. The structure of water is discussed in more detail in Chapter 4.

Hydrogen-bond formation is in all probability of great importance in the interaction between polysaccharide molecules as in cellulose and also in the interaction between peptide chains in proteins. Hydrogen bonds "zipper" these long-chain molecules together.

Hydrogen bonds can be detected by spectroscopic analysis. The stretching of the valence bond incidental to the formation of a hydrogen bond results in a decrease in the frequency of vibration, and the shift of the normal

TABLE 8

## ENERGIES AND LENGTHS OF SOME HYDROGEN BONDS

Bond	Substance	Energy in Kilocalories	Lengths in Å
O—H—O	H <sub>2</sub> O	4.5	2.76
O—H—O	CH <sub>3</sub> OH	6.2	
O—H—O	(CH <sub>3</sub> COOH) <sub>2</sub>	8.2	
C—H—N	(HCN) <sub>2</sub>	3.28	
N—H—N	NH <sub>3</sub>	1.30	3.38
O—H—N	Peptides		2.85
N—H—F	NH <sub>4</sub> F	5.0	2.63

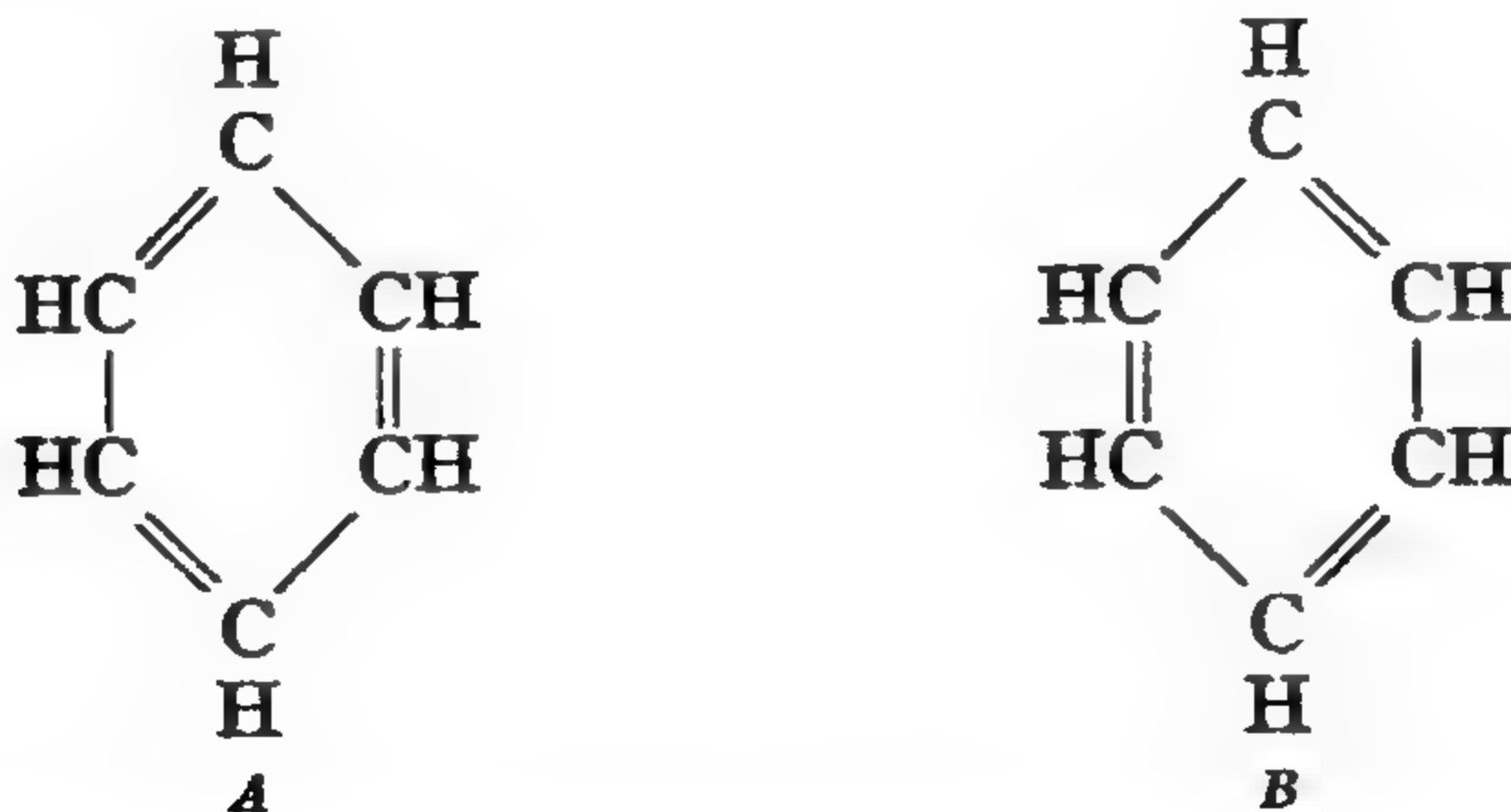
absorption spectrum of a compound is, accordingly, taken as evidence of hydrogen-bond formation. Owing to the fact that hydrogen bonds are long-distance bonds, absorption due to hydrogen bonds occurs at a low frequency.

In keeping with all chemical bonds there is an approximate inverse relation between bond strength and bond distance. Hydrogen bonds, in general, being quite weak bonds, their lengths are greater than those of covalent bonds. The energies and distances of some hydrogen bonds are shown in Table 8.

### RESONANCE

It is frequently difficult to assign to a molecule a single electronic structure which represents its properties satisfactorily. Often two or more electronic structures seem to be about equally good. In such cases it is best to say that the actual molecule resonates among the possible structures. These various resonating structures do not correspond to different kinds of molecules; there is only one kind of molecule present, with an electronic structure which is a hybrid structure of all the possible electronic structures. Resonance is to be distinguished from tautomerism. A tautomeric mixture actually has in it the different tautomeric compounds, which exist simultaneously. The essential feature of the resonance idea is that a state exists intermediate between the states represented by the resonating structures. Two or more structures do not exist; the resonating structure is the only structure present.

A resonating structure is more stable than any of the individual structures. Many molecules show resonance between two or more electronic structures, and in many cases this extra resonance stability or resonance energy, as it is called, is of crucial importance for the existence of a molecule. A classical example of resonance is shown by the benzene ring, which resonates between the two following structures.



The benzene ring is thereby stabilized and is more unreactive than would be anticipated from the possession of three double bonds.



Consider the molecule of gaseous hydrogen chloride. This molecule can exist as

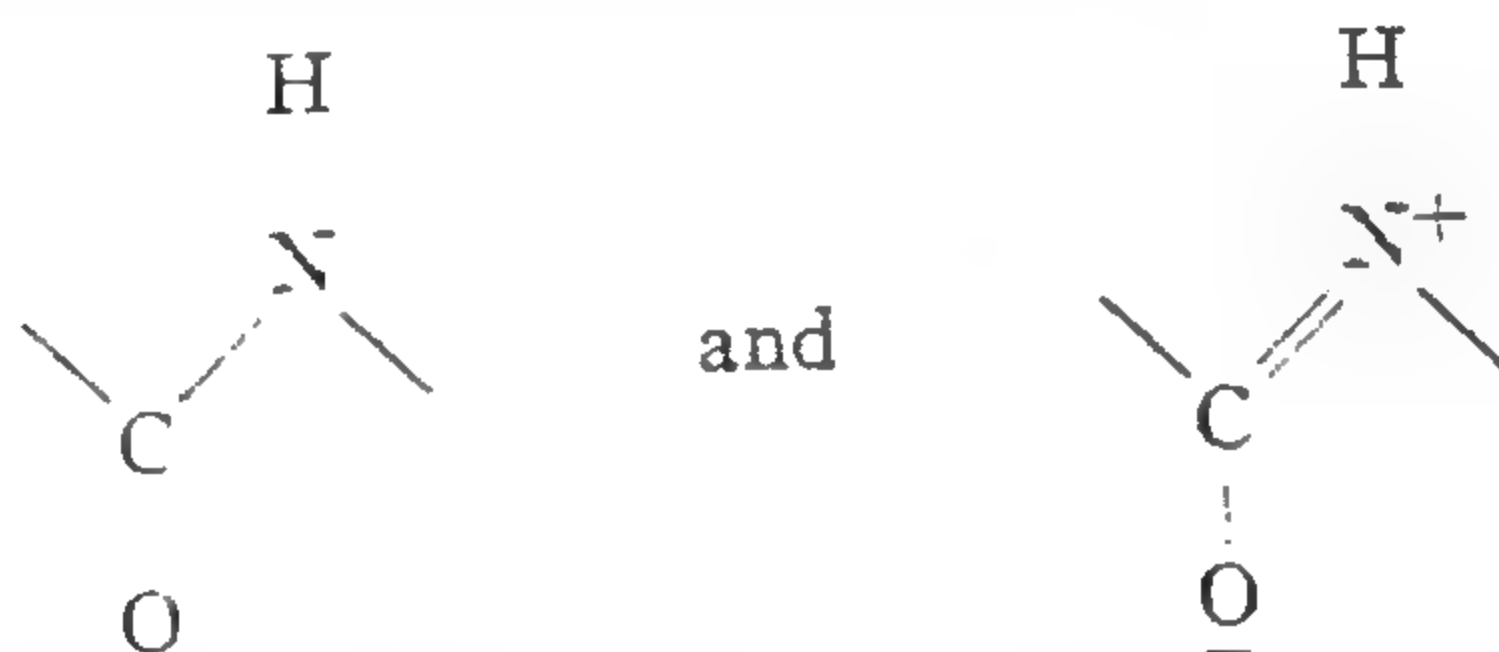


or as



That is, the bond between the hydrogen and chloride can be either an ionic one or a covalent bond and the hydrogen chloride resonates between these two structures. It is possible to calculate the approximate amount of ionic character associated with a bond from a consideration of the electronegativities of the two atoms. Such a calculation indicates that the bond in hydrogen chloride has about 17 per cent ionic character. Most chemical bonds are intermediate in nature between the extremes of a true covalent bond and a true ionic bond.

As we have seen, the distance for a single bond between nitrogen and carbon is 1.47 Å and for a double bond it is 1.27 Å. The peptide bond which is the carbon-nitrogen bond connecting amino acid residues in a protein has a length of 1.33 Å. Evidently, the peptide bond has a character intermediate between a single and a double bond. We, therefore, say that the peptide bond resonates between the structures



It is possible to calculate the percentage of double-bond character associated with a bond by means of the equation<sup>2</sup>

$$P = \frac{100 \cdot r_s - r}{2r - r_s - 3r_d} \quad (5)$$

where  $r$  is the observed length of the actual bond, and  $r_s$  and  $r_d$  are the lengths of pure single and pure double bonds. Substituting the bond distances of the peptide bond in equation 5, we find that this bond has about 50 per cent double-bond character.

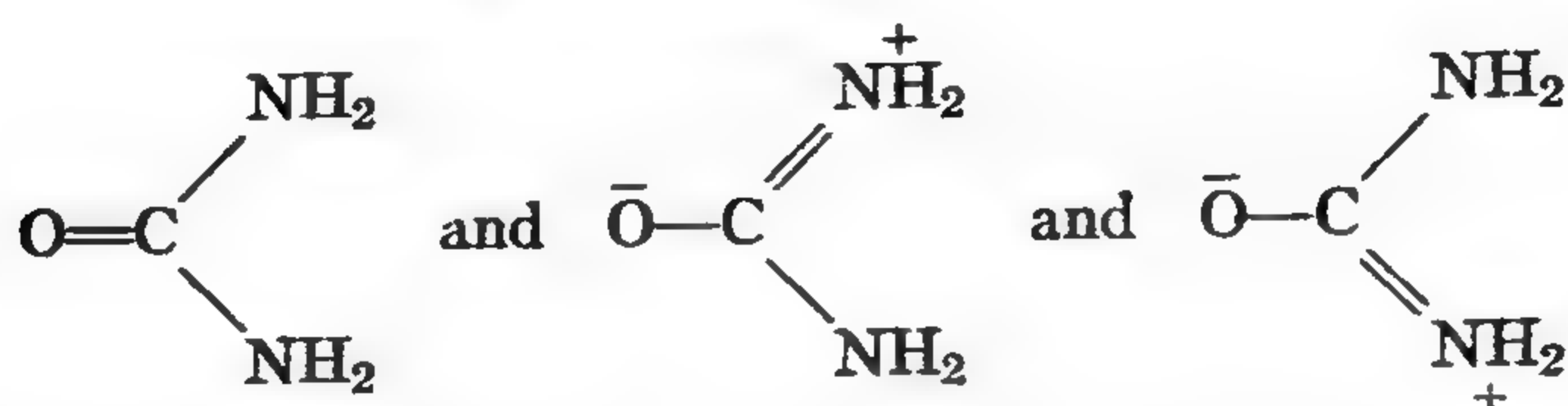
#### CALCULATION OF RESONANCE ENERGY

The simplest way to calculate the resonance energy is to calculate the heats of formation of the compound by summing the normal bond energies

<sup>2</sup> L. Pauling, *The Nature of the Chemical Bond*, Cornell University Press, Ithaca, N. Y., 1945.

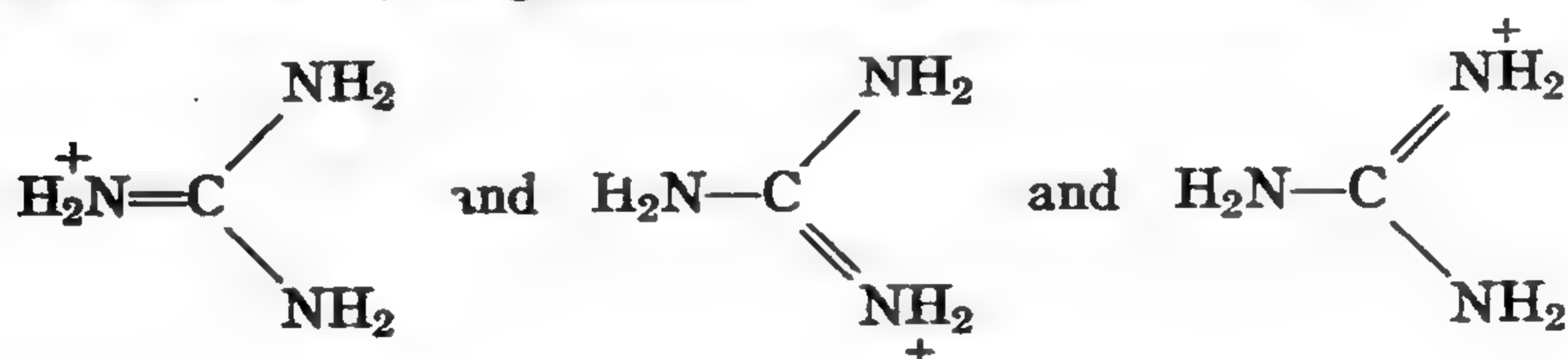
and comparing this sum with the heat of combustion of the compound. For example, the heat of formation of the benzene gas molecule from separated atoms is found from the heat of combustion (789.2 kcal. per mole) and the heats of formation of the products of combustion ( $\text{H}_2\text{O}$  and  $\text{CO}_2$ ) to have the value 1039 kcal. The sum of the bond energies ( $6\text{C}-\text{H} + 3\text{C}-\text{C} + 3\text{C}=\text{C}$ ) gives the value 1000 kcal. per mole for the heat of formation of benzene without resonance. The difference 39 kcal. per mole is the resonance energy of benzene.

Urea resonates between the structures

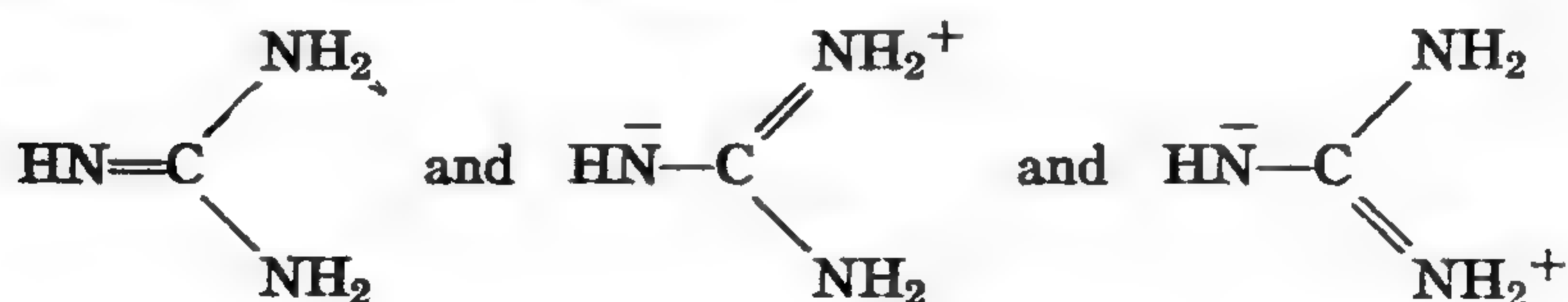


The addition of a proton to urea would destroy the capacity for resonance and explains why urea is not a strong base (the resonance energy of urea is about 37 kcal. per mole).

On the other hand, the guanidinium ion resonates between



and these structures are all equivalent; guanidine itself resonates between



which are not equivalent structures, and, therefore, the resonance energy should not be large. The difference in resonance energy between the guanidine and guanidinium ion is about 7 kcal. and explains why guanidine has such a large affinity for protons.

## DIPOLE MOMENT OF MOLECULES

There exists within many molecules a separation of electrostatic charges causing the molecule to be electrically asymmetric. A measure of the electrical asymmetry is the dipole moment ( $\mu$ ) which is equal to unit charge ( $4.80 \times 10^{-10}$  e.s.u.) multiplied by the distance separating the charges, the



distance being expressed in centimeters. Since molecular distances are of the order of  $10^{-7}$  centimeter, the dipole moment should be of the order of  $\sim 10^{-18}$ ; the quantity  $1 \times 10^{-18}$  is a Debye unit.

Dipole moments of molecules are obtained from dielectric constant studies. The dielectric constant can be defined as the ratio of the electrostatic potential existing between electrostatic charges in a vacuum to that of the medium in question. The dielectric constant of any medium is greater than unity because of polarization, i.e., the potential between two charges is always less than it is in a vacuum. Table 9 shows the dielectric constant of some common substances.

TABLE 9

DIELECTRIC CONSTANT OF SOME COMMON SUBSTANCES AT 20° C.

Substance	Dielectric Constant
Water	80
Nitrobenzene	35.8
Methanol	32.4
Ethanol	25.0
<i>n</i> -Propanol	20.81
Acetone	19.6
<i>i</i> -Propanol	18.62
<i>n</i> -Butanol	17.0
Petroleum oil	2.12
Benzene	2.28

A quantity closely related to the dielectric constant is polarization which manifests itself as a separation or displacement of charges in a medium. This displacement requires that work be done on the system and results in a decreased potential across the plates of a condenser. Polarization can be calculated from the dielectric constant by means of the Clausius-Mosotti relation which is

$$P = \frac{M}{\rho} \left( \frac{D - 1}{D + 2} \right) \quad (6)$$

where  $\rho$  is the density of the liquid or of the gas and  $M$  is its molecular weight.

Not much information can be gained from the determination of the polarization of a pure polar liquid such as water or alcohol, because there is too much interaction between the molecules. The procedure in the study of polar molecules is as follows: a small amount of the particular substance is dissolved in a non-polar solvent, i.e., a solvent whose molecular polarization is negligible, such as carbon tetrachloride, benzene or *para*-dioxane. The

dielectric constant of the dilute solution is then measured as a function of the concentration of the solute, the polarization of the solute and solvent is assumed to be additive, and, accordingly, the polarization of the solute can be calculated.

The three ways in which a substance may become polarized are:

1. The electrons within the atoms are displaced relative to the atomic nuclei.

2. The molecules are stretched, twisted, or bent by displacement of atoms within the molecules.

3. The dipolar molecules are oriented in the electrical field, the positive end of the molecule being directed towards the negative condenser plate, and the negative end towards the positive plate.

These three factors are known as the electronic, the atomic, and the molecular contributions to the dielectric constant. The electrons have a very small mass and, hence, can respond to frequencies even higher than visible light. In fact, the dielectric constant at these high frequencies is equal to the square of the index of refraction of the medium. Atoms respond to infra-red light but not to greater frequencies, and the square of the index of refraction to infra-red light is equal to the sum of the electronic and atomic contribution to the dielectric constant. The total polarization is the sum of the electronic, atomic, and molecular orientation components and

$$P = P_0 + \frac{4\pi N\mu^2}{9kT} \quad (7)$$

where  $P_0$  is the part of the polarization due to the combined effects of electrons and the atoms. The last term is the part due to molecular orientation where  $N$  is Avogadro's number,  $6.023 \times 10^{23}$ ;  $k$  is Boltzmann's constant and is  $1.38 \times 10^{-16}$  erg per degree;  $\mu$  is the dipole moment; and  $T$  is the absolute temperature.  $P_0$  is assumed equal to the square of the index of refraction of visible light. Shown in Table 10 are the dipole moments of some molecules.

The interpretation of the dipole moment in terms of molecular structure is complicated by the fact that the observed dipole moment is the resultant of all the dipoles in the molecule and each chemical bond in the molecule would be expected to make some contribution. Dipole moments being vector quantities it is not possible to sum them arithmetically.

## DIPOLE MOMENT OF ZWITTER IONS

The amino acids, proteins, and phospholipids are interesting as a class of polar molecules. Such molecules exist at the proper acidity of the solution as zwitter ions (sometimes called dipolar ions). The general form of such a

# ATOMS AND MOLECULES

TABLE 10

DIPOLE MOMENTS OF SOME MOLECULES EXPRESSED IN DEBYE UNITS

Substance	Molecular Weight	Dipole Moment
Benzene	78	0
Carbon tetrachloride	154	0
Chloroform	119	1.05
Methanol	32	1.68
Ethanol	46	1.70
Acetic acid	60	1.40
Water	18	1.85
Urea	60	8.6
Glycine	75	15
Diglycine	132	26
Triglycine	189	32
Egg albumin	45,000	250
Insulin	35,000	300
$\beta$ -Lactoglobulin	35,000	750

zwitter ion can be illustrated by the simplest of the amino acids, glycine  $\text{CH}_2\text{NH}_2\text{COOH}^-$ , shown in Fig. 1.

Such a marked separation of charge as occurs in glycine would be expected to produce a high dipole moment which can be estimated from structural considerations. The positive charge is supposed to be located at or close to

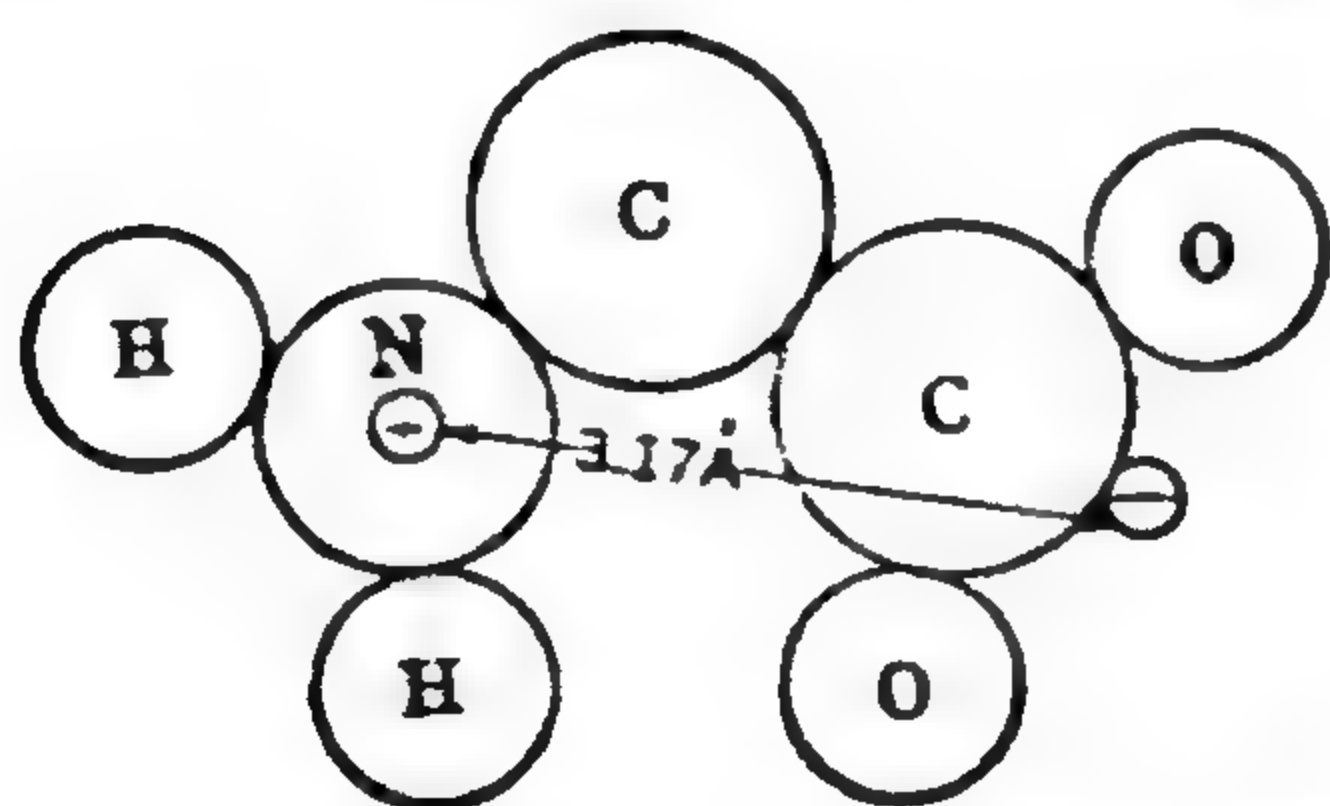


FIG. 1 Structural formula of the amino acid glycine showing distance of separation of the positive and negative charges.

the center of the nitrogen atom; the negative charge resides midway between the two oxygens of the carboxyl group. The distance between these charges is estimated to be 3.17 Å. Multiplying this distance by the electronic charge gives a dipole moment of about 15.2 Debye units.

The direct determination of the dipole moment of amino acids and of proteins by means of dielectric constant measurement is complicated by

the fact that the amino acids and proteins are, as a rule, insoluble in non-polar solvents. This necessitates the use of polar solvents such as water. The Clausius-Mossotti relation is not, however, valid for polar solvents and cannot be used to calculate polarization from dielectric constants of such solvents.

It has been found that the addition of amino acids or of proteins to water increases the dielectric constant linearly with increasing concentration.



This increase, called the molar dielectric increment, is denoted by  $\delta$  and is positive and large for all  $\alpha$ -amino acids and very nearly the same (about 23.0) and is independent of the type of polar solvent used (water-alcohol mixtures, urea-water mixtures). There is also a linear increase of  $\delta$  with increasing number of carbon atoms intervening between the  $\text{NH}_3^+$  and  $\text{COO}^-$  groups, the increment being almost exactly 13 for each additional carbon atom.

Wyman<sup>3</sup> concludes that the polarization of a solution of a zwitter ion in a polar solvent is directly proportional to the dielectric-constant increment and

$$\delta = \alpha\mu^2 - K \quad (8)$$

where  $\alpha$  is a proportionality constant and  $K$  is the decrement in the dielectric constant due to the non-polar part of the zwitter ion. Usually,  $K$  is small compared with the effect due to  $\text{NH}_3^+$  and  $\text{COO}^-$  groups and can be neglected. The value of  $\alpha$  can be estimated from structural considerations or by other means and equation 8 can thus be used to calculate the dipole moment of zwitter ions. Table 10 shows the dipole moment of amino acids, of peptides, and of proteins calculated by this method. The dipole moment of proteins appears very large, but, when account is taken of the great size of these molecules, proteins really show a high degree of electrical symmetry.

Conner, Clarke, and Smythe<sup>4</sup> studied the dielectric constant of peptide solutions as a function of the frequency of alternation of the charge on the plates of the condenser and concluded that there is a marked degree of rigidity in peptide molecules. They further conclude that, although the potential energy barriers between the various molecular configurations are relatively high, they are not prohibitive for crossing and, accordingly, there is a large measure of freedom to attain random distribution of orientation about the valence bonds. The great rigidity of the peptides probably arises at least in part from the partial double-bond character of the peptide bond which is a consequence of the resonance occurring in this bond.

At a later point in this book we shall discuss the dielectric constant as a function of frequency (Chapter 14).

## MOLECULAR ABSORPTION

Electromagnetic radiations cover a vast range of wavelengths extending from X-rays through radio waves. The velocity of an electromagnetic wave in empty space is very nearly equal to  $3 \times 10^{10}$  centimeters per second. Velocity of propagation of a wave is equal to the frequency multiplied by

<sup>3</sup> J. Wyman, *J. Am. Chem. Soc.*, 56, 536 (1934); see also *Chem. Revs.* 19, 236 (1936).

<sup>4</sup> W. P. Conner, R. P. Clarke, and C. P. Smythe, *J. Am. Chem. Soc.* 64, 1379 (1942).

the wavelength. Table 11 shows some of the characteristics of electromagnetic radiations.

Molecules absorb radiant energy of the appropriate wavelength, and absorption spectra of molecules can be classified into three types: (1) rotational spectra in which parts of the molecule rotate about valence bonds, (2) vibrational spectra in which the chemical bonds undergo stretching or bending in the sense of a spring, and (3) the electronic spectra arising from transition between electronic states.

TABLE 11  
SOME CHARACTERISTICS OF ELECTROMAGNETIC RADIATIONS

Type of Radiation	Absorbing Element	Wavelength ( $\lambda$ )	Frequency ( $\nu$ )	Kcal. Energy per Mole
X-Ray	Inner-electrons	0.006-100	$5 \times 10^{19}$ - $3 \times 10^{15}$	$4.75 \times 10^6$ -285
Ultra-violet	Valence-electrons	100-390	$3 \times 10^{15}$ - $7.7 \times 10^{14}$	285-73
Visible				
Violet	Valence-electrons	390-422	$7.7$ - $7.1 \times 10^{14}$	73-68
Blue	Valence-electrons	422-492	$7.1$ - $6.2 \times 10^{14}$	68-58
Green	Valence-electrons	492-535	$6.2$ - $5.6 \times 10^{14}$	58-53
Yellow	Valence-electrons	535-586	$5.6$ - $5.1 \times 10^{14}$	53-48.6
Orange	Valence-electrons	586-647	$5.1$ - $4.7 \times 10^{14}$	48.6-44
Red	Valence-electrons	647-770	$4.7$ - $3.9 \times 10^{14}$	44-37
Infra-red				
Near				
	Stretching	2,000-6,000	$1.5$ - $0.5 \times 10^{14}$	14-4.75
	Deformation	6,000-30,000	$5$ - $1.0 \times 10^{13}$	4.75-0.95
Far	Rotation	30,000-200,000	$10.0$ - $1.5 \times 10^{12}$	0.95-0.14

Pure rotation involves small changes in energy, and, accordingly, the wavelength of the absorbed radiation is long and occurs in the so-called far infra-red (about 200  $m\mu$ ) which is a difficult experimental region. In liquids and solids the rotation is restricted and a combination of vibration and rotation is obtained.

In general, the vibrational spectra have frequencies 10 to 100 times greater than those due to rotation, and their vibrations lie in the region in which it is easier to make absorption measurements. There are two factors that cause a vibrational frequency to be high: the small mass of the atoms participating and the high value of the restoring force. Vibrations involving the distance of separation (the valence vibrations) of atoms are higher than those due to valence angle changes which are called deformation vibrations. For quite simple molecules, it is possible to determine interatomic distances from vibrational spectra, and indeed interatomic distances involving hydrogen atoms cannot be determined accurately by other methods.

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The various valence bonds have rather characteristic frequencies of vibration, and, accordingly, it is possible to obtain information concerning the types of bonds in a molecule by the study of its infra-red absorption spectrum. In a large molecule such as a sterol, the infra-red absorption spectrum is highly elaborate with much intricate detail. Infra-red spectrometry thus provides a means of identification that is free of ambiguity. A great many sterol-like compounds have been "finger printed," and infra-red spectroscopy has been of great usefulness in the investigation of the sex hormones and hormones from the adrenal cortex.<sup>5</sup>

The measurement of the infra-red absorption spectrum is not easy. Glass cannot be used for lenses and prisms since glass is opaque to infra-red. Concave mirrors are used in place of lenses, and the prism is made of a salt such as NaCl, LiCl, etc. The solvent for the solute is also a limitation. Water, for example, has such a rich absorption spectrum of its own that it cannot be used. Studying thin films of solid deposited on strips of silver chloride has had some success. This has been the technique for the study of peptides and proteins. An interesting development in the study of the infra-red spectrum of polypeptides has been the use of polarized infra-red light. Such light shows a preferential adsorption depending on the direction of the valence bond.<sup>6</sup>

## VISIBLE AND ULTRA-VIOLET ABSORPTION

As has been noted, the absorption of radiation in the visible and ultra-violet region is due to electronic motion. In general, the problem is so complex that it is not capable of exact treatment.

The absorption spectrum of a substance is, of course, related to its color; when the absorption band is in the ultra-violet, the substance will be colorless, but, if the presence of certain groups in the molecule causes the band to shift to regions of longer wavelengths, visible color will result. Absorption spectra in the ultra-violet as well as in the visible region are thus manifestations of similar electronic transitions. The smaller the frequency, the smaller is the energy change involved in the transition; therefore, in a colored substance the optical electron must be bound in a relatively loose manner.

Most of the substances that absorb in the visible either are to some extent unsaturated or contain an atom with an incomplete inner shell of electrons. The latter is true for inorganic salts containing colored ions like chromium.

<sup>5</sup> R. F. Furchgott, H. Rosenkrantz, and E. Shorr, *J. Biol. Chem.* 163, 375 (1946); 164, 621 (1946); 167, 627 (1947).

R. N. Jones, V. Z. Williams, M. J. Whalen, and K. Dobriner, *J. Am. Chem. Soc.* 70, 2024 (1948).

<sup>6</sup> J. Ambrose and W. E. Hanbry, *Nature* 163, 483 (1949).

Here the relatively small energy difference giving rise to the color is due to a regrouping of the electrons in the inner, incomplete shell. Because of their shielded positions these electrons do not participate strongly in chemical bonding, and so a high degree of chemical stability is compatible with the presence of low excitation levels.

The color of dyes is often due to a transition between two states resulting from the regrouping of two or more electronic configurations. The frequency of this oscillation is as a rule lower if the two electronic configurations between which the oscillation takes place differ strongly from each other.

### SCATTERING OF LIGHT

The incident light induces a dipole in the atom, this dipole vibrates with the same frequency as the incident radiation and emits radiation. This mechanism of scattering changes only the direction of the propagation of the light and not its frequency. This is called the Rayleigh scattering. However, not all the scattered radiation has the same frequency as the incident light. There appear in the scattering additional frequencies which contain the natural frequency of vibration of the molecule. These frequencies correspond to the vibration frequencies of the chemical bonds and are identical with those observed in the infra-red spectrum. This type of scatter gives rise to the Raman spectrum. The Raman spectrum yields the same kind of information as does infra-red spectrography, and the experimental details are somewhat simpler and the apparatus less expensive.

### ABSORPTION OF RADIATION AND CHEMICAL REACTIONS

The first law of photochemistry states that only absorbed light can cause chemical action. The second law of photochemistry states that, in the primary photochemical process, one quantum of active light is absorbed per molecule of absorbing and reacting substance.

If  $\nu$  is the frequency of the absorbed radiation, then the corresponding quantum is  $h\nu$  and this is the amount of energy absorbed by each reacting molecule. The energy  $E$  absorbed by one mole is then  $Nh\nu$  where  $N$  is Avogadro's number, and

$$E = Nh\nu = \frac{NhC}{\lambda} \quad (9)$$

where  $C$  is the velocity of light and  $\lambda$  is the wavelength of the absorbed radiation. Inserting the values of  $N$  as  $6.023 \times 10^{23}$ ,  $h$  as  $6.61 \times 10^{-27}$ , and  $C$  as  $2.997 \times 10^{10}$ , the energy is obtained in ergs. If we divide by



$4.185 \times 10^4$  we shall have the energy expressed in kilogram calories or

$$E = 2.854 \times \frac{10^4}{\lambda} \quad (10)$$

where  $\lambda$  is to be expressed in  $m\mu$ . The quantity  $E$  for a given wavelength is sometimes referred to as one Einstein of radiation. A wavelength of 270  $m\mu$  corresponds to an energy of about 100 kilogram calories per mole; for red light with a wavelength of 750  $m\mu$  the corresponding energy absorption is approximately 38 kilogram calories. From these results it is clear that radiations in the ultra-violet portions of the spectrum should be more active chemically than those of longer wavelengths.

One mole of absorbing substance should decompose for every  $2.854 \times 10^4 \lambda$  kcal. of radiation absorbed, and the photochemical equivalent of 1 kcal. is  $\lambda \ 2.854 \times 10^4$  moles. The results of experiment are expressed in terms of the quantum yield which is defined as the number of molecules decomposed by each quantum of radiation absorbed. The quantum yield should be unity if theory is obeyed exactly. Actually, there are frequently secondary reactions associated with the primary photochemical reaction which obscure the simple correspondence between quanta absorbed and molecules decomposed.

The most important photochemical reaction is that of photosynthesis in which carbon dioxide is reduced in the chloroplast of plants by the action of sunlight. The photochemical step is



in which 112,000 calories are absorbed. At a wavelength of 660  $m\mu$  the energy per quantum is 43,000 calories. There has been considerable controversy concerning the quantum yield of photosynthesis. The early work of Warburg indicated that about 4 quanta are required per mole of oxygen produced. Warburg and Burk<sup>7</sup> have repeated this work and conclude that under favorable conditions a suspension of *Chlorella* cells will on the average produce one mole of oxygen per 4 quanta of light absorbed. Thus the energy gain is 65 per cent efficient. Doubt remains, however, regarding the interpretations of the experiments of Warburg and Burk.

An excellent review of the action of radiation on proteins is that by McLaren.<sup>8</sup>

## LIGHT ABSORPTION IN SOLUTION

Frequently, the biochemist employs light absorption as an analytical tool. When light is transmitted through a body, some portion of it is always

<sup>7</sup> O. Warburg and D. Burk, *Arch. Biochem.* 25, 410 (1950).

<sup>8</sup> A. D. McLaren, *Advances in Enzymol.* 9, 75 (1949).



absorbed. If any single wavelength of light is considered, the amount of the monochromatic light transmitted is proportional to the intensity of the incident light. Thus doubling the intensity of the incident light should double the amount transmitted and  $I_t/I_i$  is equal to some constant  $a$ , where  $I_i$  is the intensity of the incident light and  $I_t$  is the intensity of the transmitted light. The value of  $a$  is a constant for any given wavelength, but it differs with different wavelengths. If the thickness of the colored object is doubled, the incident light of the second half, having been reduced by absorption in the first half, is still further reduced in the same proportion. Thus the absorption is an exponential function of the thickness ( $d$ ) and the equation

$$\frac{I_t}{I_i} = a^d \quad (11)$$

is an expression of Lambert's law.

When the colored object is a solution, a similar law may be derived for the influence of concentration of the dissolved colored substance and

$$\frac{I_t}{I_i} = a^c \quad (12)$$

where  $c$  is the concentration of the colored substance. This is an expression of Beer's law. If Lambert's and Beer's laws are combined, we have

$$\frac{I_t}{I_i} = a^{dc} \quad (13)$$

which can be expressed in a logarithmic form as

$$\log \frac{I_t}{I_i} = -Kdc \quad (14)$$

where  $K$  is a constant under stated conditions and is termed the specific extinction coefficient when  $c$  is expressed in grams. If the concentration is expressed in moles,  $K$  becomes the molecular extinction coefficient and is usually denoted by  $\epsilon$ . Optical density ( $D$ ), another term often used, is equal to  $Kcd$ . The relation between the various terms is

$$D = Kcd = \log \frac{100}{T} = \log 100 - \log T \quad (15)$$

where  $T$  is the per cent transmission and is equal to  $100I_t/I_i$ . Evidently, a plot of the optical density against the concentration should yield a straight line the slope of which is equal to  $Kd$ .

If there is association between the molecules of the colored substance in solution, Beer's law will not be obeyed because the extent of association will, in general, be a function of the concentration of the dissolved substance and the dissociated molecules do not have the same extinction coefficient as the associated molecules. Under such circumstances, a plot of the optical density against concentration will not yield a straight line.

Klotz<sup>9</sup> has taken advantage of the shift in the wavelength of maximum absorption of dyes adsorbed on protein molecules to investigate the extent of binding of the dye molecules by the protein.

Early photometric work employed visual colorimeters which involved the matching of colors by the eye. Later, excellent photoelectric colorimeters appeared which employ photoelectric cells to measure the intensity of the transmitted light. A still sounder development has been the construction of spectrophotometers which select a very narrow wavelength band by means of a prism or diffraction grating. One of the more outstanding of these instruments is the Beckman spectrophotometer.

Three informative books on molecular structure are: *The Nature of the Chemical Bond* by L. Pauling, Cornell University Press, Ithaca, N. Y., 1945; *Electronic Interpretations of Organic Chemistry* by A. E. Remick, John Wiley & Sons, New York, 1949; *The Theory of Resonance and Its Application to Organic Chemistry* by G. W. Wheland, John Wiley & Sons, New York, 1949.

## PROBLEMS AND QUESTIONS

1. The half-life of carbon 11 is 20.35 minutes. How long would it take for 95 per cent of a sample of this isotope to disintegrate? Ans.: 87.8 minutes.

2. To a hydrolysate from 5 grams of protein are added 50 milligrams of pure  $\text{CD}_3\text{-CHNH}_2\text{COOH}$  (deuterium substituted alanine). Sixty milligrams of crystalline alanine are isolated that have a deuterium content of 1.10 per cent by weight. Calculate the percentage of the alanyl residue in the protein. Ans.: 3.96 per cent.

3. From the dimensions of the water molecule given in Chapter 4 and the dipole moment in Table 10 of Chapter 1, calculate the moment of the oxygen-hydrogen bond. Ans.: 1.51D.

4. Given three substances A, B, and C each of which shows a maximum light adsorption at different wavelengths. How would you determine the amount of each with a spectrophotometer when these substances are mixed together in solution?

<sup>9</sup> I. M. Klotz, *Chem. Revs.* 41, 373 (1947).

## Chapter

# 2

## ENERGETICS

The science of energetics (thermodynamics) deals with energy transformations. Thus, when a person walks upstairs, certain substances undergo chemical reactions in his muscles with the performance of work and liberation of heat. The work energy has increased the gravitational potential energy of the person's body, and the heat has gone into body warmth. This thermodynamic situation has been analyzed with considerable accuracy in the metabolism laboratories.

Although the metabolic studies of the physiologist are of great importance and are appropriate for what they are intended, the biochemist naturally desires a more detailed and intimate study of energy transformations than the physiologist is usually prepared to furnish. The purpose of this chapter is to present a limited exposition of the methods of chemical energetics (thermodynamics).

First, let us inquire into what we mean by the terms heat and work. Heat may be defined as that form of energy which passes from one body to another solely as the result of a difference in temperature. From a molecular point of view, heat is a disordered motion of molecules and this motion can be separated into three factors, i.e., translation, rotation, and vibration, and each type of motion contributes to the heat capacity of a body. An outstanding character of heat is the ability to induce expansion in a homogeneous object. This property is taken advantage of in the measurement of heat because, in general, the amount of expansion is directly proportional to the intensity of the heat, i.e., to the temperature.

The centigrade scale is based on the division in the temperature between the freezing and the boiling points of water into 100 equal parts. The absolute temperature is taken as directly proportional to the volume of a definite mass of an ideal gas at constant pressure or to the pressure at constant volume. It has been found that the expansion of an ideal gas is  $1/273.16$  parts of its volume at zero degrees centigrade, and, accordingly, the absolute zero where there is no molecular motion of any kind is 273.16 degrees below zero



on the centigrade scale. To obtain the degrees absolute (Kelvin scale) we add 273.16 to the degrees centigrade.

The unit of heat is the calorie, defined as the quantity of heat required to raise the temperature of one gram of water from 15° C. to 16° C. A kilogram calorie or large calorie (sometimes written Calorie) is equal to 1000 calories.

The heat capacity of a body is the property which when multiplied by the temperature change gives the quantity of heat that has entered or departed when the body is brought in contact with another body having a different temperature.

$$C = \frac{Q}{T_2 - T_1} \quad (1)$$

where  $Q$  is the quantity of heat in calories,  $C$  is the heat capacity, and  $T$  is the temperature.

Heat differs from all other forms of energy in one important respect. Whereas all other forms of energy can be, in principle, completely converted into work, heat cannot be completely converted into work without leaving some change in the system or its surroundings.

## WORK

Force causes a body to be accelerated and when the acceleration in centimeters per second per second is multiplied by the mass in grams the product is the force in dynes.

When a force of one dyne acts through a distance of one centimeter, the work in ergs is obtained. It is the work done in lifting a gram weight one centimeter divided by the acceleration of gravity (981 cm. per sec. per sec.). Energy is defined as any property that can be produced from or converted into work; heat is energy in transit.

It was early discovered that there is an equivalency between work and heat. Thus, if we perform the original experiment of Joule and measure the rise in temperature of a given weight of water as produced by the rotation of paddle wheels which are caused to rotate by a falling weight, we can obtain the equivalency. When this experiment is performed with all its modern refinements, it is found that

$$1 \text{ calorie} = 4.185 \times 10^7 \text{ ergs}$$

Knowing this result, we are in a position to state both heat and work changes in terms of calories.

## DIMENSIONS

We shall have occasion to discuss the dimensions of numerous physical quantities in the course of this book. It seems best to anticipate these discussions with a short exposition at this point.



Physical quantities can be conveniently reduced to four fundamental dimensions, length ( $L$ ), mass ( $M$ ), time ( $t$ ), and temperature ( $T$ ). For example, the dimensions of a volume are length cubed ( $L^3$ ). Some other physical quantities and their dimensions are:

$$\text{Force} = \text{Acceleration} \times \text{Mass} = \frac{LM}{t^2}$$

$$\text{Tension} = \text{Force per unit length} = \frac{M}{t^2}$$

$$\text{Pressure} = \text{Force per unit area} = \frac{M}{Lt^2}$$

$$\text{Energy} = \text{Work} = \text{Force} \times \text{Distance} = \frac{L^2M}{t^2}$$

Some rules of dimensional analysis are as follows:

1. Pure numbers, such as  $\pi$ ,  $\frac{1}{2}$ , and 50, are dimensionless.
2. Complete exponents are dimensionless, although parts of exponents may have dimensions.
3. Ratios of dimensionally identical quantities have no dimension.
4. Sines, cosines, etc., are dimensionless.
5. Differentials retain the dimensions of the variables.
6. If quantities are additive they must have the same dimensions.

Dimensional analysis is of great aid to one with a limited mathematical equipment in protecting himself against mathematical frauds. If, for example, it is found that the dimensions of a certain equation cannot be made to cancel out, then that equation is incorrect.

### ENERGY CHANGES

The energy possessed by a system falls into two categories. The internal energy is characteristic of the system itself and includes the translational energy of the moving molecules, the rotational and vibrational energy, as well as the energy of the electrons and nuclei. The energy due to the position of the system in a field of gravitational, electrical, and magnetic forces is often ignored in thermodynamic calculations, and interest is centered on the internal energy.

Usually, we deal only with energy changes and not with the total internal energy. Thus, the energy change of a system may be due to the performance of work on or by the system and also to the transfer of energy to or from the system in form of heat and

$$\Delta E = Q - w \quad (2)$$

where  $\Delta E$  is the change in internal energy,  $Q$  is the quantity of heat that has been transferred into or out of the system, and  $w$  is the work done on or by the system.

### HEAT CHANGES

If a chemical reaction is allowed to proceed, it will give off or take up heat; and if the system does no work (reaction carried out in a bomb calorimeter), the heat given off or taken up will always be the same for a given weight of the reacting materials and at a given temperature.

If we burn 1 gram-molecular weight of glucose to gaseous  $\text{CO}_2$  and liquid  $\text{H}_2\text{O}$ , we shall have 673,000 calories of heat evolved. Since heat has been given off, the system is poorer by 673,000 calories; accordingly, we speak of the negative heat of reaction. We write the reaction to indicate this:



If heat had been absorbed from the surroundings instead of being given off, it would have been considered positive.

The biochemist is usually interested in processes carried out at constant pressure instead of at constant volume, but it is experimentally easier to determine the heats of reaction at constant volume (in a bomb calorimeter). The relation between the heat at constant volume and constant pressure is

$$\Delta H = Q_v + P \Delta V \quad (3)$$

where  $\Delta H$  is the heat of the reaction at constant pressure,  $Q_v$  is the heat at constant volume, and  $\Delta V$  is the volume change at pressure  $P$ . If  $n_1$  is the number of moles of gaseous reactants, and  $n_2$  is the number of moles of gaseous products of the reaction, the process is accompanied by an increase of  $n_2 - n_1$  or  $\Delta n$  moles of gas. The increase in volume of gas is then  $V \Delta n$  where  $V$  is the volume of one mole of gas or the work done is  $P V \Delta n$ , and, since  $PV$  is equal to  $RT$ , we see that the relation 3 can be written

$$\Delta H = Q_v + RT \Delta n \quad (4)$$

The burning of glucose to liquid water and gaseous  $\text{CO}_2$  involves no change in volume so that  $\Delta n$  is zero and  $\Delta H$  in this case is equal to  $Q_v$ . In other cases where there is a net volume change, the heat at constant pressure can easily be calculated from the heat at constant volume by equation 4. The heat at constant pressure is known as the enthalpy.

The heat of reaction is equal to the difference between the heat content of the products of the reaction and the heat content of the reactants.

The heat of formation of a compound is the increase of the heat content  $\Delta H$  when one mole of the substance is formed from its elements at a given temperature and pressure. The heat contents of all elements in their



standard states is assumed to be zero, and, since the heat of formation of a compound is the difference between the heat content of the compound and that of the elements, it follows that the heat content of a compound is equal to its heat of formation.

### WORK CHANGES

If we could build a machine which was completely efficient and which would allow us to utilize all the energy of a chemical reaction to perform work, we would find that upon oxidizing 1 gram-molecular weight of glucose to gaseous  $\text{CO}_2$  and liquid  $\text{H}_2\text{O}$ , we could realize as a maximum 688,160 calories of work at constant temperature and pressure. The term free energy is applied to such work energy; it means that so much energy is free and not bound and can be obtained from the system, provided that we have the proper apparatus for accomplishing it. The same conventions of sign apply to free energy changes as for heat changes. Thus, in the oxidation of 1 gram-molecular weight of glucose at  $25^\circ \text{C}$ ., free energy is evolved and accordingly its sign is negative.



If it had been necessary to do work on the reaction to make it proceed, the free energy change would have been positive. No actual machine could obtain 688,160 calories of work from the burning of a gram-molecular weight of glucose because no machine is 100 per cent efficient. We could, in fact, calculate the efficiency of our machine by comparing its actual performance with the theoretical. A much more useful purpose, however, to which free energy data can be put is the prediction of the spontaneity of a process. Evidently, if the free energy change for a given reaction is positive, we must do work on the system before it will react and such a reaction will not proceed of its own accord. Such reactions are known as endergonic reactions. If, on the other hand, the free energy change is negative, the reaction may proceed spontaneously. We shall see presently how we can use the magnitude of the free energy change to predict the extent of the reaction. Reactions with a negative free energy are called exogonic reactions.

There are certain limitations to the usefulness of predictions based upon free energy changes. In an isolated system we can say definitely that, if the free energy change is positive, the reaction cannot occur. In a living system, however, we are not dealing with a single isolated system but with a multitude of interrelated systems, and the necessary free energy may be supplied by other chemical reactions. Another limitation is the time factor. A reaction with a negative free energy may proceed so slowly as to have no practical significance.

## ENTROPY CHANGES

We have noted that, in the burning of a gram-molecular weight of glucose, 673,000 calories of heat are evolved, whereas the maximum amount of work which can be obtained from the reaction is 688,160 calories. There is an apparent discrepancy of 15,160 calories. The question of the source of these 15,160 calories arises. The answer is that, during the completely efficient oxidation of glucose by the machine, 15,160 calories of heat flowed into the reaction mixture from the outside and were converted into work. That is, if we had conducted our reaction in an isolated container in such a fashion that no heat could flow into the mixture to maintain the temperature constant, we would have found that as the machine worked the temperature dropped. This heat, which flows into or out of a system at constant temperature and pressure while the reaction is allowed to proceed in such a fashion as to obtain its maximum efficiency, is sometimes known as the reversible heat of reaction. It receives this name because a reaction which proceeds in such a fashion as to yield its maximum efficiency must proceed in a reversible manner at every point in its course. It is evident that for the burning of glucose in the manner we have described

$$\Delta H = \Delta F + \text{Reversible heat of reaction} \quad (5)$$

It has been found that equation 5 is true of all reactions and all processes carried out at constant temperature and pressure.

The entropy change  $\Delta S$  is closely related to the reversible heat of reaction and

$$\Delta S = \frac{q}{T} \quad (6)$$

where  $q$  is the reversible heat of reaction and  $T$  is the absolute temperature. Thus, the entropy change involved in the reversible oxidation of 1 mole of glucose at 25° C. is 15,160/298 or is 50.8 cal. per degree per mole.

When an ideal gas is allowed to expand in a reversible manner against a piston from a volume  $V_1$  to a volume  $V_2$ , the work done by the expansion is

$$\text{Work} = \int_{V_1}^{V_2} P dV = RT \int_{V_1}^{V_2} \frac{dV}{V} = RT \ln \frac{V_2}{V_1} \quad (7)$$

where  $R$  is the gas constant into whose nature we shall presently inquire. The total energy of the system remains constant, and, accordingly, at constant temperature the work done is equal to the heat absorbed by the gas and

$$\frac{q}{T} = \Delta S = R \ln \frac{V_2}{V_1} \quad (8)$$



Entropy is related to the probability of an event occurring. Thus if  $P_1$  is the probability associated with one situation and  $P_2$  that with a second situation, the entropy change in proceeding from the first to the second situation, provided that there is no net energy change, is given by

$$\Delta S = R \ln \frac{P_2}{P_1} \quad (9)$$

What we really mean, therefore, by the statement that the entropy must increase in a spontaneous reaction is that the reaction mixture must proceed towards a more probable state. Entropy is a measure of the number of possible configurations of a system having a given energy.

A simple example of a process involving a change of entropy which is related to the degree of randomness is that occurring during the melting of a solid. Consider ice converted into water at constant temperature and pressure. The molar latent heat of fusion of ice is 1436.37 calories. The entropy change is, therefore,  $1436.37/273.16$  or  $5.26EU$ , and the ratio of the number of possible configurations of a mole of liquid water molecules to that of one mole of ice water molecules is given by equation 9. We find upon substituting the numerical values that  $P_2/P_1$  is 13.96.

Pauling<sup>1</sup> has calculated the entropy of an ice crystal from the number of possible configurations that the water molecule in ice can assume.

One of the outstanding features of living matter is its high degree of order. This order is maintained at the expense of the environment by the metabolism of food stuffs taken into the body. As Schrödinger<sup>2</sup> has put it, an organism feeds on negative entropy.

### FREE ENERGY CHANGE AND THE EQUILIBRIUM CONSTANT

Our main object in discussing the free energy change or the maximum work obtainable from a process is to be able to predict the occurrence and extent of chemical reactions. It is necessary, therefore, to show that a relation exists between the free energy change of a reaction and its equilibrium constant. The relation between these two quantities is by no means obvious, and it is not an easy one to visualize. Its importance for the continuity of our argument is so great, however, that an attempt will be made to outline the relation between these two factors. This is not intended to be a rigorous derivation.

Suppose that we have a large living cell whose membrane is freely permeable to water. The cell is to be surrounded by an aqueous medium consisting of dilute solutions of substances A and B. The cell has in its interior

<sup>1</sup> L. Pauling, *J. Am. Chem. Soc.* **57**, 2680 (1935).

<sup>2</sup> F. Schrödinger, *What Is Life?* The Macmillan Co., New York, 1945.

an enzyme which is capable of converting  $A$  into  $B$  or  $B$  into  $A$  so that these two substances are at all times in equilibrium with each other inside the cell. The cell now proceeds to accumulate a certain volume of the aqueous medium in a vacuole and then concentrates the solution in respect to  $A$ , so that its concentration is exactly equal to that in the cytoplasm of the cell. Of course, osmotic work is performed by the cell in carrying out this step. Then the cell, without doing additional work, delivers this small volume of solution of  $A$  to the cytoplasm, where it reacts to produce  $B$ .

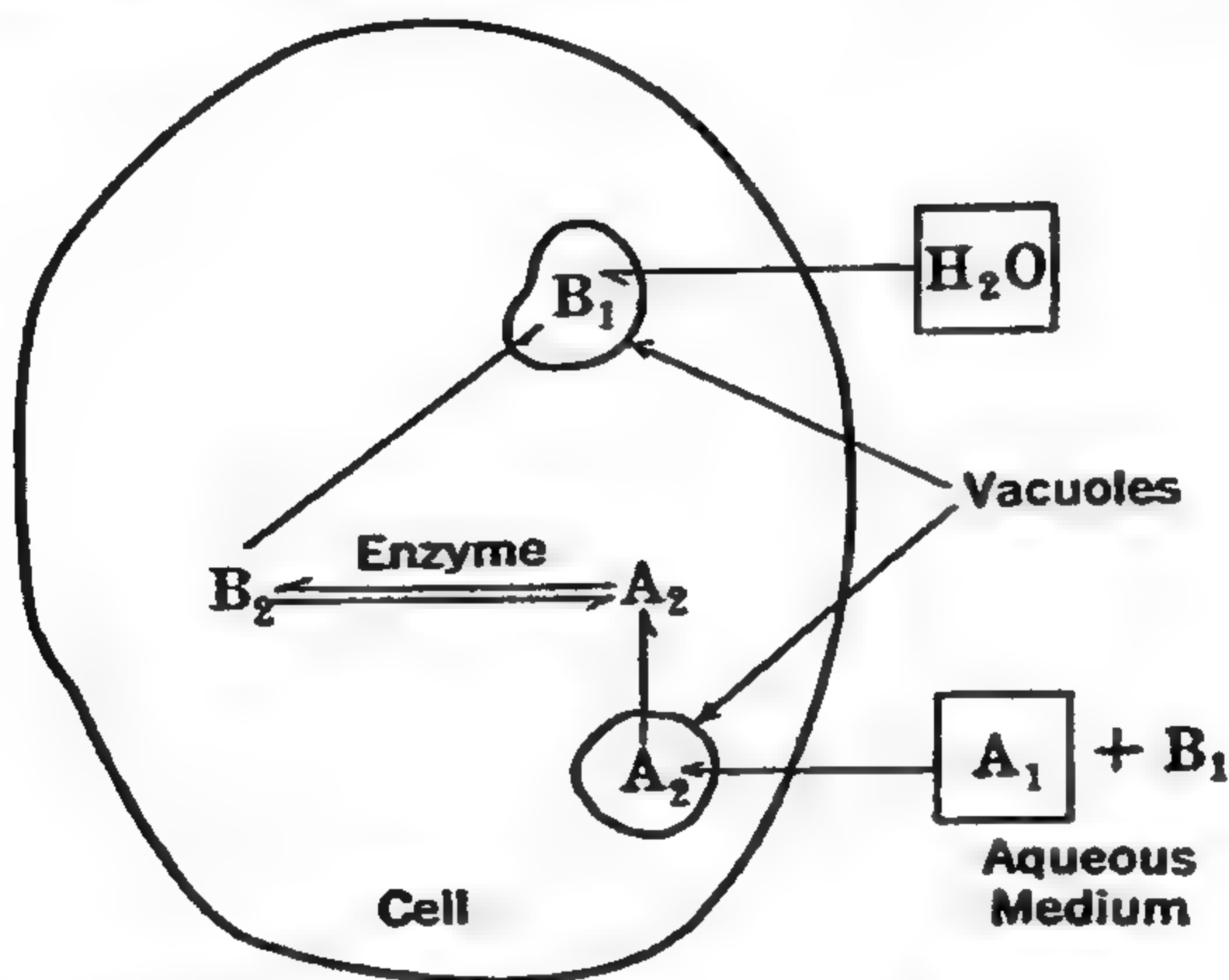


FIG. 1. Diagrammatic representation of living cell doing osmotic work.

The excess of  $B$  thus produced is withdrawn by the cell into a second vacuole, where it has the same concentration in respect to  $B$  as it has in the cytoplasm. No work is involved in this step. The cell is now able to do osmotic work by allowing water from the aqueous medium to diffuse into the vacuole containing  $B$ , until the concentration of  $B$  in the vacuole is the same as that in the aqueous medium outside the cell. The problem is to calculate the osmotic work done by the cell in taking  $A$  from the aqueous medium where its concentration is  $A_1$ , into the cell where its concentration is  $A_2$ , and the osmotic work gained by the cell in allowing the molecules of  $B$  produced from  $A$  to be diluted from  $B_2$  to  $B_1$ ,  $B_1$  being the concentration of  $B$  in the outside, aqueous medium. The difference between these osmotic-work terms is the work required to convert  $A$  into  $B$ .

The osmotic work done in concentrating  $A$  is given by

$$\text{Work}_A = RT \ln \frac{A_2}{A_1} \quad (10)$$

while the work gained by the cell in diluting  $B$  is

$$\text{Work}_B = RT \ln \frac{B_2}{B_1} \quad (11)$$

## ENERGETICS

The net work is equal to the difference between these terms; accordingly

$$\text{Net work} = RT \ln \frac{A_2}{A_1} - RT \ln \frac{B_2}{B_1} \quad (12)$$

Rearranging terms, and remembering that since the cell is assumed to be completely efficient the free energy change is equal to the net work, we have

$$\Delta F = -RT \ln \frac{B_2}{A_2} + RT \ln \frac{B_1}{A_1} \quad (13)$$

Since  $A_2$  and  $B_2$  are in equilibrium

$$\frac{B_2}{A_2} = K \quad (14)$$

and therefore

$$\Delta F = -RT \ln K + RT \ln \frac{B_1}{A_1} \quad (15)$$

If the concentrations of  $A$  and of  $B$  are chosen equal to unity, we have

$$\Delta F_0 = -RT \ln K \quad (16)$$

This equation is a completely general one for a system at constant temperature and pressure, and it is valid no matter how many reacting substances are present, provided that the reactants are initially at unit concentration. We shall find later in this chapter that thermodynamic concentrations must be used, in order that this equation may hold for all concentrations.

Consider again a mixture made up of substances  $A$  and  $B$  in equilibrium with each other and imagine a series of reactions and corresponding equilibrium constants. We then calculate the free energy change as a function of these equilibrium constants. We further set  $A$  plus  $B$  equal to 100 per cent and express the equilibrium constants in terms of the per cent composition in respect to  $B$ . This permits us to plot the free energy change as a function of the amount of  $B$  expressed as per cent of the mixture. Such a plot is shown in Fig. 2 at 25° C.

The constant  $R$  in the above equations is the molar gas constant; it is given by the equation

$$R = \frac{PV}{T} \quad (17)$$

At standard temperature (273.16° absolute) and pressure (76.0 cm. of mercury), a gram-molecular weight of an ideal gas occupies 22,412 cc.

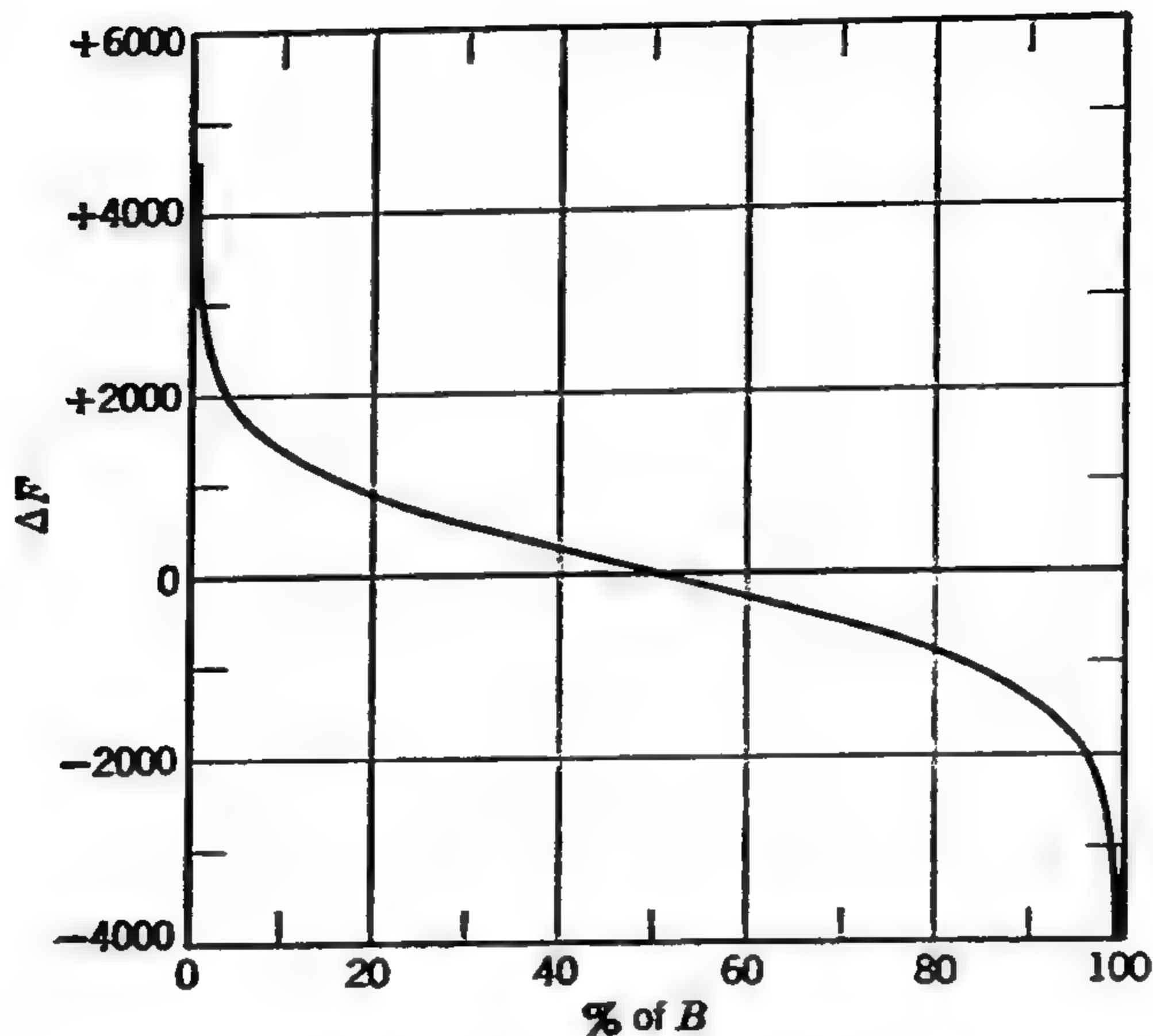


FIG. 2.  $\Delta F$  as a function of the per cent composition in respect to  $B$  at  $25^\circ \text{C}$ .

Accordingly

$$R = \frac{76.0 \times 13.596 \times 980.6 \times 22,412}{273.16}$$

$$= 8.315 \times 10^7 \text{ ergs per degree per mole}$$

Since as we have seen

$$1 \text{ calorie} = 4.185 \times 10^7 \text{ ergs}$$

we have

$$R = 1.987 \text{ calories per degree per mole}$$

Converting equation 16 to logarithms to the base 10 and substituting the value of  $R$ , we have

$$\Delta F_0 = -4.575T \log K \quad (18)$$

where  $T$  is expressed as the absolute temperature.

## EQUILIBRIUM

This term has a very precise thermodynamic meaning. When a system is in equilibrium, its capacity for doing work is zero; the free energy of the system is at a minimum. Its entropy is at a maximum; it is in its most probable state.



## DISTRIBUTION OF ENERGIES

It can be shown by the equipartition principle that the kinetic energy of a molecule can be assigned to different kinds of molecular movements. The kinetic energy of translation, for example, can be divided into that along the  $x$ ,  $y$ , and  $z$  axes, each being equal to  $\frac{1}{2}RT$  calories per mole. The total translational energy is, therefore,  $\frac{3}{2}RT$  calories per mole. It is inherently improbable that all the molecules of a given kind in a system would have

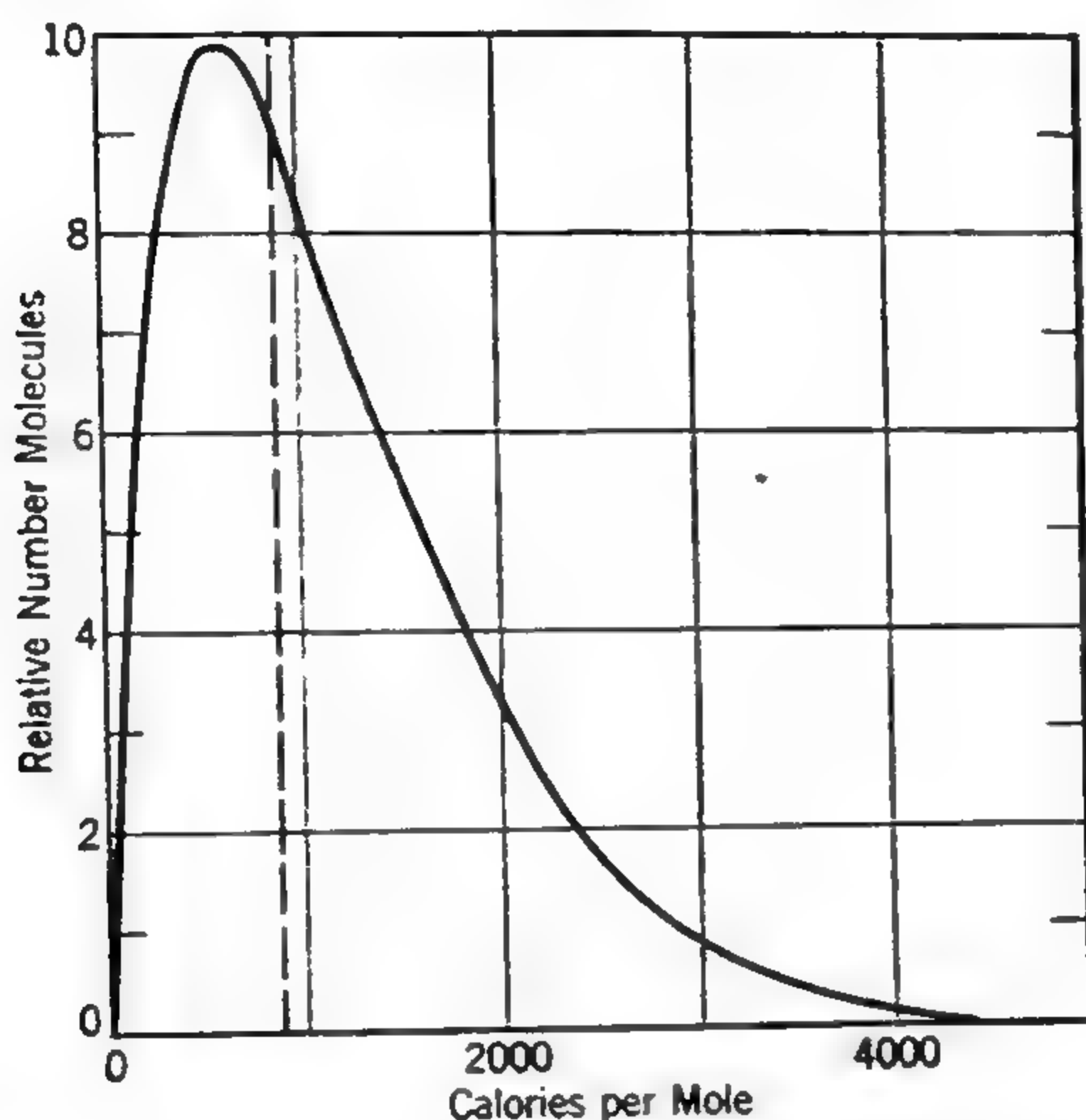


FIG. 3. Maxwell distribution of translational energies of molecules at 25° C. expressed in calories per mole. Vertical line indicates average energy per mole.

identically the same velocities, energies, etc. The distribution of velocities and energies of molecules at a given temperature can be calculated by Maxwell's distribution law.<sup>3</sup> Figure 3 shows the relative number of molecules having a particular kinetic energy of translation, the energies being expressed in calories per mole at 25° C. The average energy is also indicated as a broken vertical line. Note that the distribution of energies is skewed towards the lower energies. This skewness is more pronounced at lower temperatures. With increasing temperature the distribution curve becomes flatter and more normal in shape (approaches a Gaussian distribution curve).

The rotation of a cigar-shaped molecule about its long axis does not influence the heat capacity, but rotation about its short axis in either of two

<sup>3</sup> S. Glasstone, *Textbook of Physical Chemistry*, D. Van Nostrand and Co., New York, 1946.

directions at right angles to each other contributes  $\frac{1}{2}RT$  calories per mole so that the rotational energy of such a molecule is  $RT$  calories per mole.

The kinetic energy of a molecule depends only on the temperature and not at all on the medium, i.e., at a given temperature the kinetic energy is the same whether the molecule exists as a gas or in solution.

The Maxwell treatment gives the distribution of velocities among molecules when in a field of uniform force. The Boltzmann distribution, on the other hand, considers the distribution of molecules in a non-uniform field of force, such, for example, as the distribution of molecules of air in the gravitational field of the earth. The Boltzmann equation is derived from a consideration of the rate of transfer of molecules from a region of high field of force to one of less force and back again. At equilibrium these two rates are equal, and the Boltzmann equation is obtained by equating these rates. The distribution equation is

$$\frac{n_1}{n_2} = e^{-\psi/RT} \quad (19)$$

where  $n_1$  is the number of molecules per unit volume at one point,  $n_2$  is that at a second point, and  $\psi$  is the difference in potential energy of the molecules in the two volumes. The difference in potential energy may arise as the result of any form of force acting on the molecules, such, for example, as a centrifugal force or an electrical force acting on ions.

#### RELATION BETWEEN HEAT AND FREE ENERGY CHANGES

As we have seen, for any isothermal process at constant pressure the relation between the free energy change, heat change, and entropy change is given by the equation

$$\Delta H = \Delta F + T \Delta S \quad (20)$$

In other words, it is only when the entropy change is zero that the heat of reaction is equal to the free energy change, and it is, therefore, only under this condition that the heat of reaction is a true measure of the driving force of a process. Under all other conditions the heat change is without direct significance in predicting the extent or spontaneity of a reaction.

By combining in the appropriate manner the necessary mathematical statements of thermodynamics, the relation between the heat and free energy changes at constant pressure is obtained. This relation, known as the Gibbs-Helmholtz equation, is

$$\Delta F - \Delta H = T \left( \frac{d \Delta F}{dT} \right) \quad (21)$$

If the differential  $d \Delta F/dT$  is zero, i.e., the free energy change is independent of temperature,  $\Delta F$  will equal  $\Delta H$  of the reaction.

If we integrate the Gibbs-Helmholtz equation, assuming  $\Delta H$  to be constant, there results

$$\frac{\Delta F_2}{T_2} - \frac{\Delta F_1}{T_1} = - \frac{\Delta H(T_2 - T_1)}{T_1 T_2} \quad (22)$$

which allows us to calculate the free energy change as a function of temperature, provided that we know the heat of reaction.

By substituting equation 16 into the Gibbs-Helmholtz equation and carrying out certain mathematical manipulations, we have the van't Hoff equation

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2} \quad (23)$$

If we again assume  $\Delta H$  to be constant and integrate the van't Hoff equation, at the same time converting the natural logarithm to logarithm to the base 10 and substituting the numerical value for  $R$ , we have

$$\log \left( \frac{K_2}{K_1} \right) = - \frac{\Delta H}{4.575} \left( \frac{T_1 - T_2}{T_2 T_1} \right) \quad (24)$$

where  $K_1$  is the equilibrium constant at temperature  $T_1$  and  $K_2$  that at temperature  $T_2$ . This equation allows us to calculate  $\Delta H$  of the reaction if we know  $K_2$  and  $K_1$  or allows us to predict  $K_2$  if we know  $K_1$  and  $\Delta H$ .

Another form of the integrated van't Hoff equation is

$$\log K = - \frac{\Delta H}{4.575T} + C \quad (25)$$

where  $C$  is an integration constant.

Evidently, if  $\log K$  is plotted against  $1/T$ , the slope of the straight line will be  $-\Delta H/4.575$ , from which  $\Delta H$  may be obtained directly.

#### THERMODYNAMICS OF SOLUTIONS

Since the biochemist deals very largely with solutions instead of with solids or gases, it will be profitable to consider briefly some of the energy relations in solutions.

The analytical chemist expresses concentrations in terms of the number of moles of a given substance in 1 liter of solution, 1 mole of a substance dissolved in 1 liter of solution being a molar solution. The physical chemist, on the other hand, often finds it more convenient to express concentrations in terms of the number of moles per 1,000 grams of solvent. One mole of the substance dissolved in 1,000 grams of the solvent is known as a molal solution. The advantage of this mode of expression is that concentration is independent of temperature, which is not true of molar solutions.



A still more fundamental method of expressing concentrations is by means of mole fractions. The mole fraction of one component is equal to the total number of moles present (solvent and solutes) divided into the number of moles of the constituent in question. That is,

$$N_1 = \frac{n_1}{n_1 + n_2} \quad (26)$$

and

$$N_2 = \frac{n_2}{n_1 + n_2} \quad (27)$$

where  $N_1$  is the mole fraction of one constituent,  $n_1$  is the number of moles of that constituent present,  $n_2$  is the number of moles of the second constituent, and  $N_2$  is the mole fraction of the second constituent. This method of expression is to be preferred because thermodynamic properties which vary with concentration are proportional to the number of particles present and the mole fraction is the ratio of the number of molecules of a substance to the total number of molecules present.

Let us consider a molal solution of sucrose in water and compare these three methods of expressing concentrations. To produce a molal solution of sucrose we dissolve a gram-molecular weight of sucrose (342.17 grams) in 1,000 grams of water. The density of such a solution at 25° C. is 1.2417. Evidently the total volume of this solution is 1,342.17 divided by the density, or 1,081.08 cc. The molar concentration is, accordingly, 1.000 divided by 1,081.08, or it is 0.925 molar. The mole fraction of sucrose is (using equation 27) 0.0177.

It should be noted that the mole fraction of a solute in 1 molal aqueous solution is always 0.0177.

Although, as pointed out above, the mole fraction is a more fundamental unit of concentration than either molarity or molality, it has been the custom of physical chemists to use molality; accordingly, in what is to follow, we shall employ molality as the method of expressing concentrations. It is evident that, in the relatively dilute solutions such as the biochemist is likely to encounter, molality is almost exactly proportional to mole fraction, and we lose very little significance by using molality instead of mole fractions.

## ACTIVITY

The thermodynamist finds himself compelled to deal with another kind of concentration. It was early discovered that thermodynamic equations, such as 16 previously given, are not exact expressions except in very dilute solutions. As the concentrations are increased, such equations, in general,



fail to be exact descriptions of experimental results. The term *activity* or thermodynamic concentration was introduced to take the place of the molality concentrations. It might be said that the activity of a substance is the concentration the substance apparently has as judged by its chemical effects. More precisely, activity may be defined as being equal to the concentration, as the concentration approaches zero. This is the same thing as saying that the solute approaches ideal behavior as the solution becomes more and more dilute.

The three general causes for the departure of solutions from ideal behavior are: (1) interaction between the solute molecules (such as due to electrical charges); (2) interaction between the solute and solvent (hydration); and (3) interaction between solvent molecules. We shall have occasion to deal with these factors in considerable detail in the course of this book.

The activity coefficient is the expression for the ratio of the activity to the concentration; i.e.,

$$\text{Activity coefficient } (\gamma) = \frac{\text{Activity}}{\text{Concentration}} \quad (28)$$

The correct expression for the free energy change in proceeding from unit activity of the reactants to a condition of equilibrium for a chemical reaction of the type



is

$$\Delta F_0 = -RT \ln K = -RT \ln \frac{(\text{Activity } C)^c \times (\text{Activity } D)^d}{(\text{Activity } A)^a \times (\text{Activity } B)^b} \quad (29)$$

#### FREE ENERGY OF DILUTION

If a solution behaved in an ideal fashion, the free energy change in going from concentration  $C_1$  to  $C_2$  would be

$$\Delta F = RT \ln \frac{C_2}{C_1} \quad (30)$$

For any actual solution

$$\Delta F = RT \ln \frac{A_2}{A_1} \quad (31)$$

where  $A_1$  and  $A_2$  are the activities.

An important thermodynamic situation arises in respect to a saturated solution of a substance in contact with some of its solid phase. Since the solid is in equilibrium with the dissolved substance, the free energy change in going from the solid to the dissolved state is zero and, accordingly, the molar free energy of the substance in solution is equal to that of the solid. Free energy tables frequently give values for the solid in some definite crystalline state. The problem of finding the free energy of a substance in solution at any given concentration, therefore, consists in adding to the

free energy of the solid substance the free energy of dilution experienced in going from the saturated solution to the activity in question.

Activities can be determined in a variety of ways such as by the measurement of vapor pressure, electromotive force of the proper electrical cells, freezing-point lowering, boiling-point raising, and osmotic pressure. We shall have occasion to deal in detail with some of these types of measurements in the course of this book.

### PARTIAL MOLAR QUANTITIES

If a mole of a solute is dissolved in a very large quantity of the solution—so large a volume that the concentration is not significantly changed—the resulting increase in volume is known as the partial molar volume of the added material. In the same way we have partial molar quantities of any extensive property such as the heat content or the entropy. The partial specific volume is the increase in volume resulting from the addition of 1 gram of a solute to a large volume of a solution.

Partial molar quantities can be evaluated by graphical methods. For example, to determine the partial molar volume of a component of a solution, plot the total volume of the system against the number of moles of this component, keeping the molar number of all other components constant; the slope of the curve will give the partial molar volume of the component at the selected concentration.

An important partial molar quantity is the partial molar free energy which is equal to  $dF/dn$ , i.e., to the rate of change of the free energy of a given component in respect to the rate of change of the number of moles of that component; the temperature, pressure, and the number of moles of the other components of the solution to remain constant. The partial molar free energy was called the chemical potential by Gibbs and is usually denoted by the symbol ( $\mu$ ). It is a necessary condition for equilibrium that the chemical potential of any component be the same throughout the entire system in the same sense that the temperature must be uniform throughout a system before the system can be said to be at equilibrium. For example, if we dissolve iodine in carbon tetrachloride and shake the carbon tetrachloride solution with water, at equilibrium the chemical potential of the iodine in the carbon tetrachloride must be equal to that in the water, in spite of the fact that the concentrations of iodine in the two phases are very different.

The chemical potential is closely related to activity. The chemical potential of any component in a solution is given by

$$\mu = \mu_0 + RT \ln A \quad (32)$$

where  $A$  is the activity of the component in question and  $\mu_0$  is its chemical potential in the standard state.

If the standard state of a substance is the same in two phases in contact in equilibrium, the activity of the substance as well as the chemical potential of the substance must be identical in the two phases at any concentration.

Any change in the total free energy of a solution may be considered to result from the sum of the changes of any extensive property of the system and we can write for a solution

$$dF = \left( \frac{dF}{dT} \right)_{P, n_1, n_2} dT + \left( \frac{dF}{dP} \right)_{T, n_1, n_2} dP + \left( \frac{dF}{dn_1} \right)_{T, P, n_2} dn_1 + \left( \frac{dF}{dn_2} \right)_{T, P, n_1} dn_2 \quad (33)$$

where the subscripts indicate that these particular functions are to be considered constant.  $dF/dn_1$  and  $dF/dn_2$  are quite evidently the chemical potentials of the two components. For a system at constant temperature and pressure equation 33 may be written

$$dF = \mu_1 dn_1 + \mu_2 dn_2 \quad (34)$$

If equation 34 be integrated, we have

$$F = \mu_1 n_1 + \mu_2 n_2 \quad (35)$$

Now if equation 35 be differentiated, we have

$$dF = \mu_1 dn_1 + n_1 d\mu_1 + \mu_2 dn_2 + n_2 d\mu_2 \quad (36)$$

Comparing equation 34 with equation 36, there results

$$n_1 d\mu_1 + n_2 d\mu_2 = 0 \quad (37)$$

Equation 37 is the well-known Gibbs-Duhem equation which relates the chemical potential of one component of a solution to the chemical potential of all other components. The implication of this equation is rather far reaching. In an osmotic pressure determination of a protein (see Chapter 12), what we really measure is the activity of the water, and yet from such a measurement we calculate the activity of the protein in solution. It is because of the relation shown in equation 37 that such a calculation is possible.

#### FREE ENERGY OF FORMATION AND ITS USE

Four general methods have been used to determine the magnitude of the free energy changes in chemical reactions:

1. The measurement of the equilibrium constant  $K$  in a reversible reaction and the calculation of  $\Delta F$  by the relation  $\Delta F_0 = -RT \ln K$ .



2. The determination of the reversible electromotive force  $E$  of an electrical cell involving the reaction in question, in which case  $\Delta F = -NFE$  ( $N$  is the number of equivalents,  $F$  is a constant, and  $E$  is the electromotive force).

3. The determination of heat capacities down to very low temperatures and the utilization of these results together with other thermal data to calculate  $\Delta F$  by means of the third law of thermodynamics.

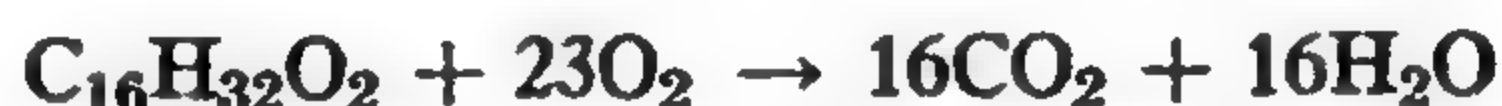
4. The combination of suitable chemical equations with known  $\Delta F$  values to give the reaction in question.

As indicated in a previous section of this chapter, the calculation of the free energy change by the first of the above methods involves the equilibrium constant and, accordingly, for precise work a knowledge of the activities of the reacting molecules. It is also necessary to be certain that the reaction under consideration is a reversible one and that equilibrium is attained within a reasonable time. These conditions limit the applicability of this method of evaluating  $\Delta F$ .

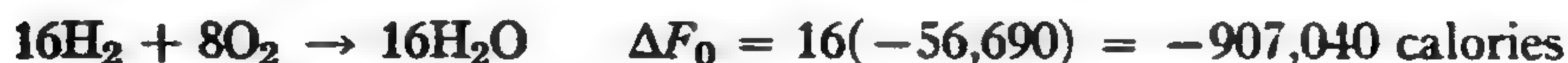
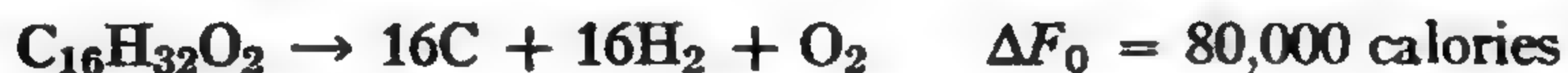
The second of the above methods will be considered in the chapters on electromotive force cells and on oxidation-reduction.

A consideration of the third method is beyond the scope of this book.

The fourth method, the combining of suitable chemical equations with known  $\Delta F$  values, is available to everyone, and we shall now attempt to illustrate it. For example, consider the burning of palmitic acid to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . We have



Looking up in Table 1 the free energy of formation of palmitic acid,  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , we have



Adding these three chemical equations together, we have the equation for the burning of palmitic acid. Adding also the free energy changes of these reactions, we have the free energy change involved in the combustion of palmitic acid, which turns out to be  $-2,338,240$  calories per mole. Evidently the burning of palmitic acid is thermodynamically a spontaneous process and will proceed to completion with a rich yield of energy.

Usually free energy tables give the free energy of formation of compounds in the solid, liquid, or gaseous states. The free energy of formation means the free energy of the hypothetical reaction whereby the compound in question is synthesized from the elements in their standard state. The free

# ENERGETICS

TABLE 1

FREE ENERGIES OF FORMATION AT 25° C. AND AT ATMOSPHERIC PRESSURE

Name	$\Delta F_0$	Name	$\Delta F_0$
Acetaldehyde		cation (aq)	-124,200
(g)	-32,000	L-Cysteine	
(l)	-31,880	(s)	-82,480
Acetic acid		(aq)	-81,630
(g)	-91,230	cation (aq)	-83,960
(l)	-94,500	anion (aq)	-70,270
(aq)	-96,210	L-Cystine	
acetate (aq)	-89,720	(s)	-166,630
Acetone		(aq)	-162,080
(g)	-36,500	monovalent cation (aq)	-164,880
(l)	-37,220	divalent cation (aq)	-166,300
d-Alanine		monovalent anion (aq)	-151,170
(s)	-88,780	divalent anion (aq)	-137,190
(aq)	-89,130	Diethyl ether	
cation (aq)	-92,320	(g)	-28,090
anion (aq)	-75,910	(l)	-28,300
Ammonia		Dulcitol (S)	-223,100
NH <sub>3</sub> (g)	-3,940	Ethyl alcohol	
NH <sub>3</sub> (aq)	-6,330	(g)	-38,600
NH <sub>4</sub> OH (aq)	-62,990	(l)	-40,200
NH <sub>4</sub> <sup>+</sup> (aq)	-18,960	(aq)	-41,850
n-Amyl alcohol (l)	-39,100	Ethyl acetate	
n-Amyl butyrate	-74,900	(g)	-76,360
L-Aspartic acid		(l)	-77,600
(s)	-175,440	Ethyl n-butyrate (l)	-76,000
(aq)	-172,890	Formaldehyde	
cation (aq)	-175,480	(g)	-26,100
monovalent anion (aq)	-167,940	(aq)	-31,020
divalent anion (aq)	-154,990	Formic acid	
n-Butyric acid (l)	-91,500	(g)	-82,520
n-Butyl alcohol	-40,400	(l)	-85,300
Calcium ion (aq)	-132,700	(aq)	-88,110
Carbon dioxide (g)	-94,450	Fumaric acid	
Carbon monoxide (g)	-33,010	(s)	-156,700
Carbonic acid		(aq)	-154,860
(aq)	-148,810	monovalent anion	-150,660
HCO <sub>3</sub> <sup>-</sup> (aq)	-140,490	divalent anion	-144,620
CO <sub>3</sub> <sup>2-</sup> (aq)	-126,390	d-α-Glucose (s)	-215,000
Chloride ion (aq)	-31,330	d-β-Glucose (s)	-215,400
Creatinine		d-Glucose (aq)	-217,020
(s)	-7,550	d-Glutamic acid	
(aq)	-7,560	(s)	-174,800
cation (aq)	-14,160	(aq)	-172,500
Creatine		cation (aq)	-175,360
(s)	-121,810	monovalent anion (aq)	-166,950
(aq)	-120,560	divalent anion (aq)	-154,030

# FREE ENERGY OF FORMATION AND ITS USE 45

TABLE 1

FREE ENERGIES OF FORMATION AT 25° C. AND AT ATMOSPHERIC PRESSURE (Continued)

Name	$\Delta F_0$	Name	$\Delta F_0$
Glycerol (l)	-113,600	Oxalic acid	
Glycine		(s)	-165,900
(s)	-88,920	monovalent anion (aq)	-164,380
(aq)	-89,570	divalent anion (aq)	-158,660
cation (aq)	-92,720	Palmitic acid	
anion (aq)	-76,350	(s)	-80,000
n-Hexyl alcohol (l)	-38,100	(l)	-78,560
Hippuric acid		Pentacosane	
(s)	-90,440	(s)	13,400
(aq)	-88,140	(l)	15,000
anion (aq)	-82,940	Phosphoric acid	
Hydrocyanic acid		H <sub>3</sub> PO <sub>4</sub> (aq)	-270,000
(g)	28,670	H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> (aq)	-267,100
(aq)	27,280	HPO <sub>4</sub> <sup>2-</sup> (aq)	-257,270
anion (aq)	39,140	PO <sub>4</sub> <sup>3-</sup> (aq)	-240,970
Hydrogen ion (aq)	0	Potassium ion (aq)	-67,430
Hydrogen peroxide (l)	-28,230	Potassium chloride (s)	-97,550
Hydroxide ion (aq)	-37,585	n-Propyl alcohol (l)	-40,900
Isopropyl alcohol (l)	-44,000	Sodium ion (aq)	-62,590
d,l-Lactic acid (l)	-124,400	Succinic acid	
L-Leucine		(s)	-178,800
(s)	-83,750	(aq)	-178,510
(aq)	-82,800	monovalent anion (aq)	-172,780
cation (aq)	-85,990	divalent anion (aq)	-165,090
anion (aq)	-69,710	Sucrose (s)	-371,600
Malic acid (s)	-149,400	1-Tyrosine	
L-Malic acid		(s)	-97,640
(s)	-211,450	(aq)	-94,090
(aq)	-213,570	cation (aq)	-97,130
divalent anion (aq)	-201,850	anion (aq)	-81,610
Mannitol (s)	-222,200	Urea	
Methyl alcohol		(s)	-47,410
(s)	-38,890	(aq)	-49,010
(l)	-39,960	Water	
n-Octyl alcohol (l)	-35,100	(g)	-54,636
		(l)	-56,690

energy of the elements in their standard state is arbitrarily set equal to zero so that, in the reaction



which evolves 80,000 calories of free energy, we blame the entire energy transformation on the palmitic acid and call it the free energy of formation of palmitic acid.



It is to be remembered that the free energy change of any reaction is equal to the algebraic sum of the free energies of all the reactants involved. The reactants on the left side of the chemical equation have their signs changed previous to summation; those on the right side of the equation remain unchanged.

In order to find the free energy of a compound in solution at unit activity (standard solution state), we must add to the free energy of formation of the compound its free energy of dilution from its saturated solution to unit activity. Frequently, the activities of the substance in solution are not known, and, as a first approximation, the concentrations may be used instead of activities. In dealing with a substance miscible with water in all proportions, a convenient point of departure is the substance in the pure state. Its mole fraction in this state is unity. The free energy change for the transfer of 1 mole of the solute from this state to a very large volume of an aqueous solution in which 1 mole is dissolved in 1000 grams of water is (for an ideal solution)

$$\Delta F = RT \ln 0.0177 \quad (38)$$

If the substance ionizes in solution, we must also include the free energy of ionization. It is evident that free energy calculations for reactions in solution can be rather involved.

Table 1 is a collection of free energy data. Those free energies marked (aq) refer to a hypothetical 1 molal ideal solution or, in other words, an aqueous solution in which the activity of the solute is unity. Those marked (g) refer to a gas at standard conditions; those marked (s) refer to solid, crystalline conditions.

### THERMODYNAMICS IN BIOLOGY

As pointed out in the beginning of the chapter, metabolism studies are of a thermodynamic nature; implicit in them is the assumption of the validity of the law of the conservation of energy.

The application of some of the considerations of thermodynamics to biological systems has certain important limitations. One must be very careful about calculations which assume a state of equilibrium to exist. A point in question is the application of the Donnan equilibrium equations to the general problem of the accumulation of electrolytes by living cells. As shown by Stewart<sup>4</sup> and others, the accumulation of electrolytes by many cells involves the expenditure of energy by the cell and is in no sense an equilibrium condition; and the use of equations based upon the assumption of an equilibrium condition is manifestly unjustified. There is no doubt

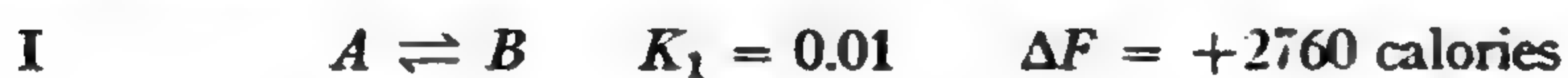
<sup>4</sup> F. C. Stewart. *Trans. Faraday Soc.* 33, 1006 (1937).

that, in some respects, living cells may be in equilibrium with their surroundings. For example, many cells appear to attain true osmotic equilibrium in a remarkably short time.

The question sometimes arises whether or not a living system is able of its own accord to proceed away from a condition of equilibrium instead of towards it, as is demanded by thermodynamics. Suppose, as a matter of argument, we were to assume that a living cell membrane could show one-way permeability to some chemical compound, say glucose; i.e., the glucose could enter the cell by simple diffusion and the cell could, by doing no work, block the exit of glucose molecules. Evidently in time the cell would build up a considerable concentration of glucose within its interior and it could use this excess concentration to do osmotic work although the cell itself had done no work in the process of accumulating the glucose. Such a situation would completely violate thermodynamic calculations made on the cell. Thermodynamics states that a membrane can never show one-way permeability without doing the required osmotic work.

### METABOLISM AND ENERGETICS

A living system has to engage in a variety of activities. It is called upon to accumulate ions and molecules against concentration gradients and to secrete against concentration gradients. It must synthesize proteins, esters, and carbohydrates, as well as many other substances all of which require the expenditure of energy. These so-called "endergonic" reactions must be coupled with an "exogonic" reaction which is capable of yielding the necessary energy. Consider



Reaction I will proceed from left to right until the concentration of  $B$  is less than one per cent of  $A$ . Reaction II will proceed from left to right until the concentration of  $B$  is less than 0.1 per cent of  $C$ . If these two reactions proceed consecutively, reaction II will rid the system of  $B$  almost completely as it is formed in reaction I. The overall reaction is



for which we have

$$K = K_1 K_2 = 10; \quad \Delta F = 2760 - 4140 = -1380 \text{ calories}$$

The overall reaction will thus proceed until the concentration of  $C$  is ten times that of  $A$ . The net result is that the free energy of reaction II has been transferred to reaction I, enabling it to proceed in a forward direction.

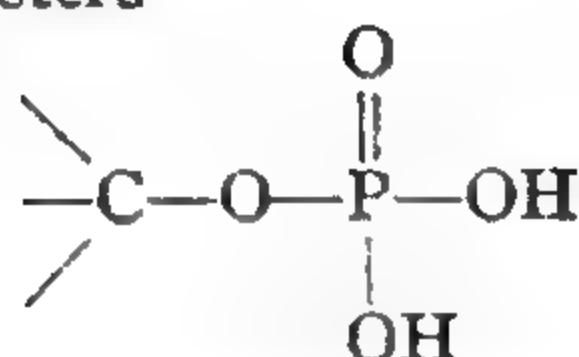
This type of coupling is, apparently, the principal one available to a living system.

The general coupling agent used by a living system is the high energy phosphate bond. Phosphoric acid ( $\text{H}_3\text{PO}_4$ ) can combine with a variety of organic substrates, and in some cases the hydrolysis of such phosphate bonds involves only moderate negative free energy changes, whereas other phosphate bonds involve much larger negative free energy changes. This last type of phosphate bond is called a high-energy phosphate bond. Table 2 gives some examples of such bonds.

TABLE 2

## FREE ENERGIES OF HYDROLYSIS OF PHOSPHATE BONDS

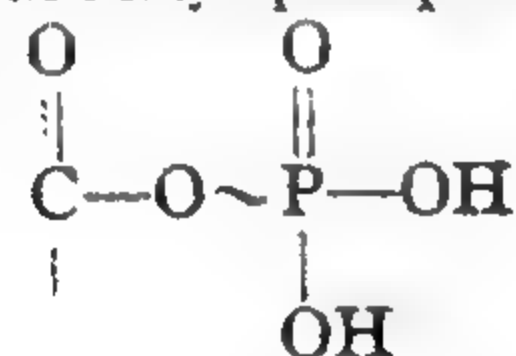
## Esters



Glycerol phosphate .

-2,300 calories

## Carbonyl phosphates



Phosphoacetic acid

-10,000 calories

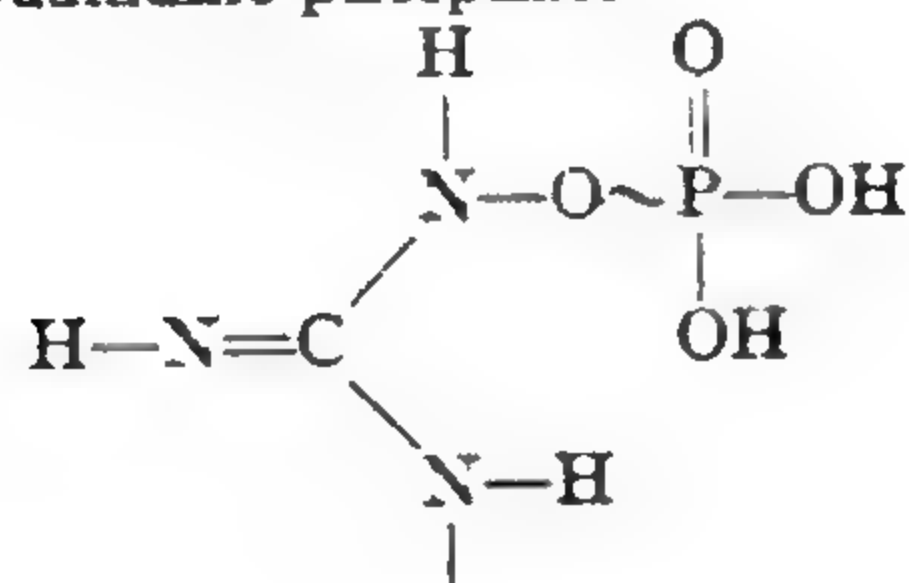
Phosphoenol pyruvic acid

-11,000 calories

1:3 Diphosphoglyceric acid

-11,000 calories

## Guanidine phosphate



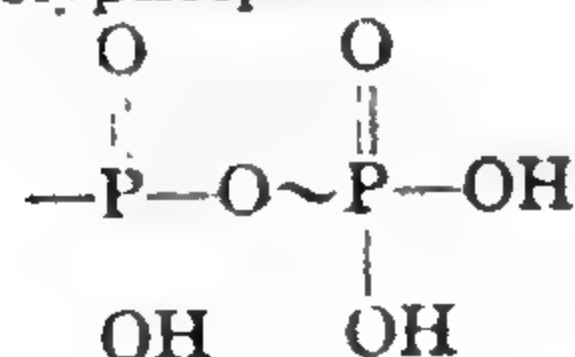
Creatine phosphate

-10,000 calories

Arginine phosphate

-10,000 calories

## Polyphosphates



Adenosine diphosphate

-10,000 calories

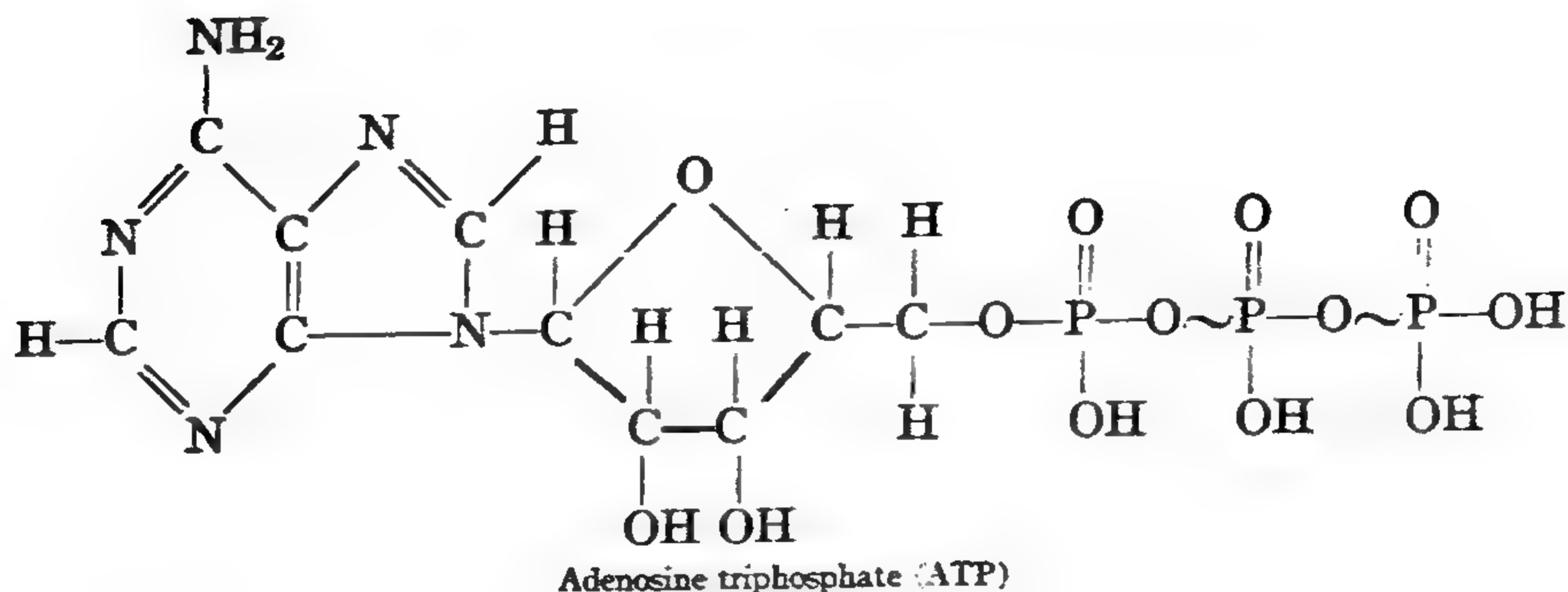
Adenosine triphosphate

-10,000 calories

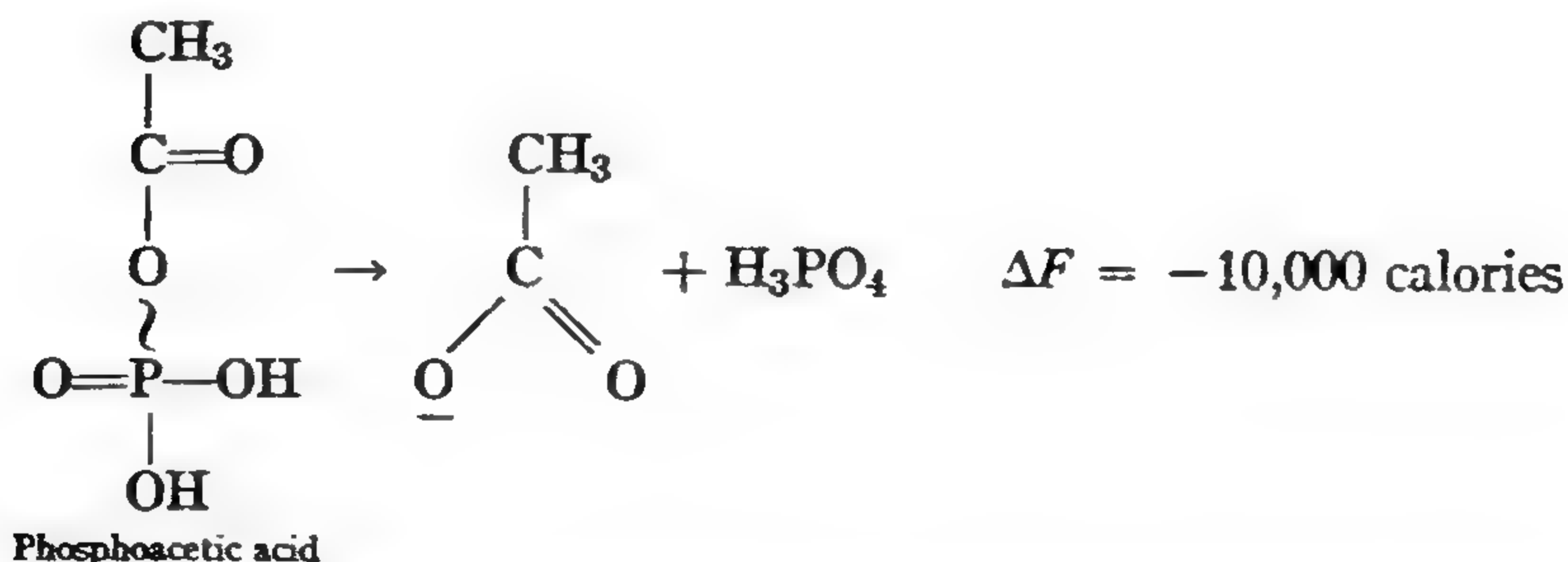
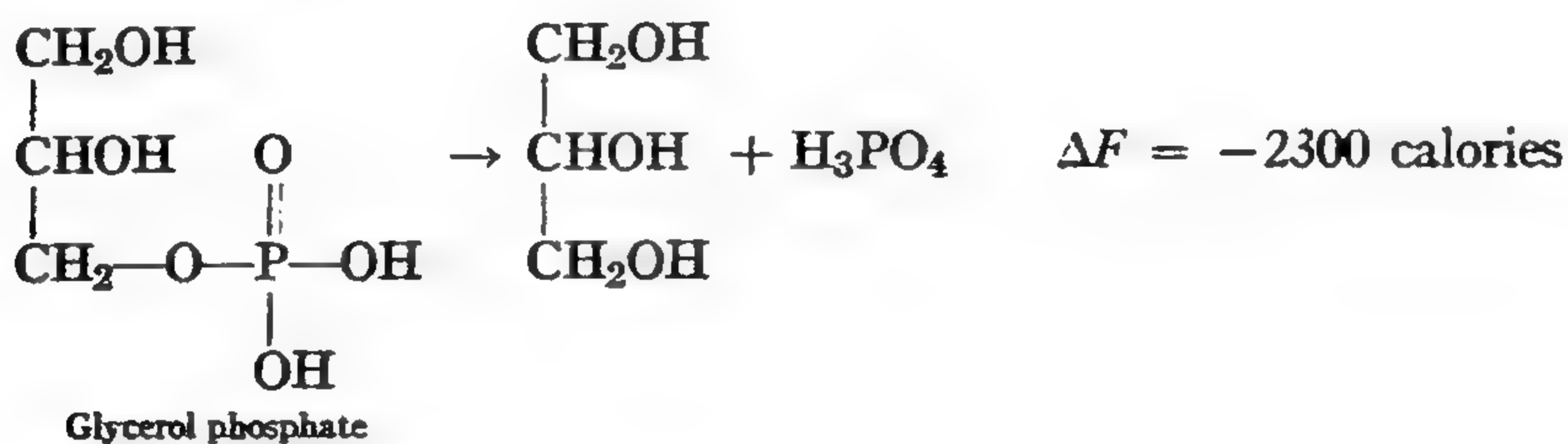
Biochemists use the term "high-energy bond" in an entirely different sense from the physical chemist when he talks about bond energies. The biochemist means that when such a bond is hydrolyzed the reaction will go very nearly to completion (the free energy change is large and negative).

The most versatile of these high-energy phosphate bonds is that occurring in adenosine triphosphate (ATP)





Several questions naturally arise concerning phosphate bonds. One of these is why some phosphate bonds hydrolyze with a large negative free energy, whereas others hydrolyze with a much more moderate yield. Consider, for example, the ester phosphate bond as contrasted with the carbonyl phosphate bond.



The acetic acid resonates with an energy of about 28 kilocalories, and, accordingly, we expect the reaction to go nearly to completion; the glycerol formed from the hydrolysis of glycerol phosphate does not resonate, and, accordingly, the energy yield for it is not nearly so large. It is not possible to account quantitatively for the energy yield of phosphate bonds, but it is possible to predict from structural considerations whether a given phosphate bond will be a high or a low energy bond. See, for example, the paper by Oesper.<sup>5</sup>

<sup>5</sup> P. Oesper, *Arch. Biochem.* 27, 255 (1950).

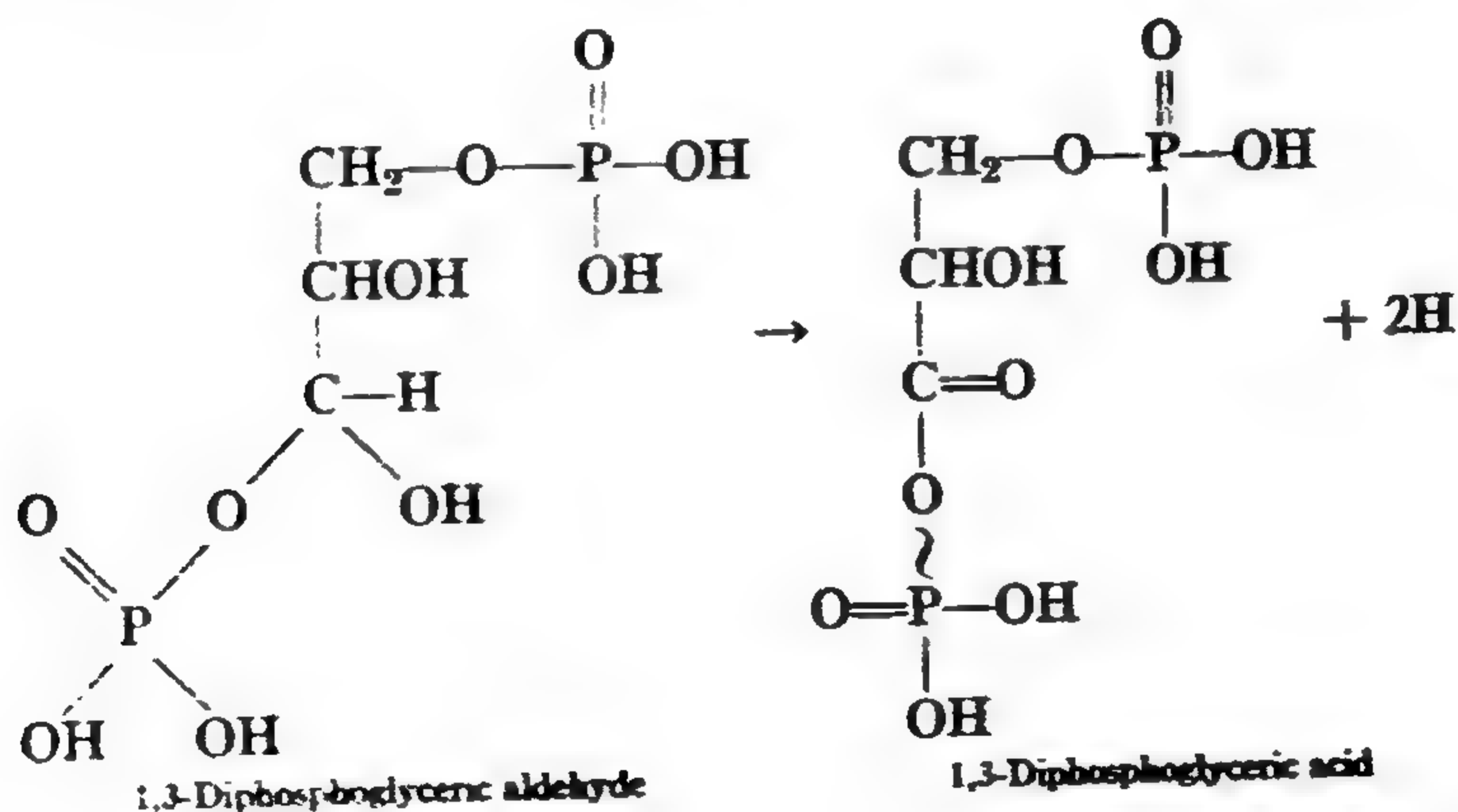
Another important question concerns the mechanism by which a living system produces high-energy phosphate bonds. A complete answer to this question involves the intricacies of cellular metabolism; it is not our purpose to enter into the details of this complex problem, but it is profitable to view the overall picture.

The living system derives its energy principally from the oxidation of fats, of carbohydrates, and of proteins. Let us center our attention briefly on the metabolism of carbohydrates.

Glycogen, which is a condensation product of glucose, is stored in the liver. The intracellular synthesis and breakdown of glycogen follow paths that are quite different from those of digestive hydrolysis. The degradation is not, in fact, a process of hydrolysis but one of phosphorolysis in which the glucosidic bonds are not broken by the elements of water ( $\text{HO—H}$ ) but by those of phosphoric acid ( $\text{HO—P}$ ) and the product of breakdown is  $\alpha$ -glucose-1-phosphate. This type of phosphorolysis involves a comparatively small free energy change and can be easily driven in either direction, depending on the concentration of glucose and of phosphate.

The  $\alpha$ -glucose-1-phosphate undergoes a series of complex reactions known as glycolysis which do not require oxygen and are known as anaerobic oxidation. During glycolysis one high-energy phosphate bond is destroyed and four are synthesized so that the net gain is three high-energy phosphate bonds, and 2 moles of lactic acid are produced for each mole of  $\alpha$ -glucose-1-phosphate oxidized.

During the course of glycolysis a high-energy phosphate bond arises when 1,3-diphosphoglyceric aldehyde is oxidized to 1,3-diphosphoglyceric acid.

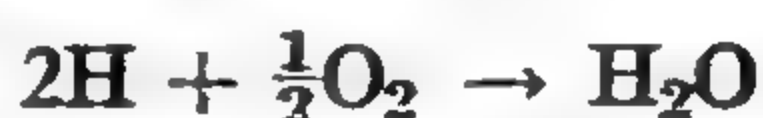


Thus, a high-energy phosphate bond is produced by the removal of two hydrogens (oxidation). The high-energy phosphate bond can then be transferred to ADP to give rise to ATP.

The free energy change (per glucose equivalent) when glycogen is oxidized anaerobically to lactic acid is, at the concentrations prevailing in tissue, about 50,000 calories. In this process there is a net gain of 3 high energy phosphate bonds, and, if each phosphate bond is worth about 10,000 calories, the process is about 60 per cent efficient in the production of high-grade, easily utilizable energy.

In aerobic metabolism of glucose, the glucose first undergoes glycolysis to pyruvic acid which is the oxidation product of lactic acid. Pyruvic acid enters what is known as the tricarboxylic acid cycle (Kreb's Cycle) consisting of a number of successive oxidations involving the removal of hydrogen from the substrates with the simultaneous creation of energy-rich phosphate bonds. The hydrogens are passed along through a series of oxidation-reduction enzymes and finally combined with oxygen to yield water.

In the complete aerobic oxidation of glycogen, the free energy change is about 720,000 calories per glucose unit and the maximum energy yield is nearly 15 times greater than for glycolysis. Apparently, about 50 high-energy phosphate bonds are produced in the complete aerobic oxidation of glycogen to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . It seems probable that every dehydrogenation can give rise to an energy-rich phosphate bond. Since the ultimate fate of the hydrogen that has been removed from the substrate is to combine with oxygen to form water, the final reaction is



This is the reaction that makes possible the existence of higher forms of life.

Excellent discussion of cellular metabolism is given by Baldwin<sup>6</sup> and by Lardy.<sup>7</sup> A clearly and simply written book on the general subject of thermodynamics is that by Klotz.<sup>8</sup> This book is highly recommended.

## PROBLEMS AND QUESTIONS

1. Calculate the gain in potential energy expressed in calories when a man weighing 80 kilograms walks up a flight of stairs, the top level of which is 10 feet above the bottom.

Ans.: 572 calories.

2. Using Table 1 of Chapter 7, calculate the molar change in free energy, in the heat, and in the entropy involved in the ionization of water at 25° C.

Ans.:  $\Delta F = 19,090$ ;  $\Delta H = 13,680$ ;  $\Delta S = -18.1$ .

3. The activity coefficient of a 0.05 molal NaCl solution at 25° C. is 0.821 and that of a one molal NaCl solution is 0.658. Dilute the molal solution to a concentration of 0.05 molal with water and calculate the free energy change per mole of NaCl.

Ans.:  $\Delta F = -1640$  calories.

<sup>6</sup> E. Baldwin, *Dynamic Aspects of Biochemistry*, Cambridge: At the University Press, 1947.

<sup>7</sup> H. A. Lardy, *Respiratory Enzymes*, Burgess Publishing Co., Minneapolis, 1949.

<sup>8</sup> I. M. Klotz, *Chemical Thermodynamics*, Prentice-Hall, Inc., New York, 1950.



4. Calculate the free energy of oxidation of one gram of *d*-glucose at 25° C. and at 35 mm. Hg oxygen tension in a 0.001 molal solution to liquid water and bicarbonate ions and at pH 7.4.

*Ans.: -3830 calories.*

Note: This is a difficult problem but, if you solve it, you have a good grasp of the subject.

5. Write down the various resonating structures of the phosphate ion  $\text{HPO}_4^{2-}$ . Explain what bearing these structures have on the problem of high-energy phosphate bonds. If possible, compare your answer with Reference 5 (Oesper).

## REACTION KINETICS

In many respects chemical kinetics is of more importance to the biochemist than thermodynamics. Biochemical processes can, in general, occur in a variety of ways; i.e., the free energy relations permit a number of possible reactions arising from a given situation. The relative speeds of the various reactions determine which reaction will be the predominating one.

The speed of a reaction will evidently depend on a number of factors, such as the specific nature of the reacting molecules, the concentrations, the temperature, the presence or absence of appropriate enzymes, and the nature and condition of the medium in which the reaction takes place.

Since reaction kinetics deals with the speed of chemical reactions, it is essential that we understand clearly what we mean by the speed or velocity of a chemical reaction. We must also know in what manner the reaction velocity is to be measured and expressed.

### REACTION VELOCITY

The velocity of a chemical reaction is the instantaneous rate of disappearance of a particular molecular species at any given time. It is possible to express this concept graphically. If the concentration is plotted as ordinate against the time as abscissa, then the slope of the line at any given time is equal to the velocity of the reaction at that time. Unfortunately, this velocity so estimated varies, in general, with the concentration and, accordingly, is of limited significance in the analysis of the kinetics of a reaction. We must find a method by which velocity can be referred to a standard concentration. There are three methods for accomplishing this:

(a) The determination of the order of the reaction and the evaluation of the velocity constants. The standard state of reference being unit concentration for a first-order reaction and for a second-order reaction is the condition when the product of the concentrations of the two reacting species is unity.

**A.** The determination of the amount of reactant changed in a given time state, after the effect of the reaction. The standard state then becomes the initial concentration of the reactants.

**C.** Comparison of the reciprocals of the times required to effect a given change. Here the standard state is again the initial concentrations of the reactants.

### ORDER OF A REACTION

It is important to realize that before we can express reaction velocity in terms of velocity constants, we must first be able to formulate the kinetics of a particular reaction. For a monomolecular reaction where one molecular species is undergoing change, the velocity is simply proportional to concentration of this species.

$$\text{Velocity of reaction} = \frac{dc}{dt} = -Kc \quad (1)$$

If this equation be integrated and converted to logarithms to the base 10, we have

$$\log \frac{C_0}{C} = \frac{Kt}{2.3} \quad (2)$$

where  $C_0$  is the initial concentration and  $C$  is the concentration at any time  $t$ . If the reaction in question follows the kinetics of a monomolecular reaction, the plot of  $\log C_0/C$  against  $t$  will yield a straight line whose slope is  $K/2.3$ .

It should be noted from equation 2 that the magnitude of  $K$  is independent of the manner in which the concentration is expressed.  $K$  has the dimensions of reciprocal time and is usually expressed in reciprocal seconds. Actually, most, if not all, biochemical reactions which follow the kinetics of a monomolecular reaction are really bimolecular. If, in a bimolecular reaction, one of the molecular species is in great excess, so that a decrease in its concentration is relatively slight, as compared to that of the other molecular species which is at a much lower concentration, the velocity of the reaction will be principally dependent on the concentration of the molecular species at the lower concentration, and, accordingly, the reaction will appear to be a monomolecular one. The acid hydrolysis of sucrose is such a reaction. In spite of the fact that the reaction is really bimolecular, it shows the kinetics of a monomolecular one. The velocity constant calculated on the basis of a monomolecular reaction is, however, a true measure of its velocity. All reactions that follow the kinetics of a monomolecular reaction are known as first-order reactions regardless of how many species of reacting molecules are involved.



If two molecules react, whether they are two molecules of the same compound or molecules of two different compounds, the reaction is known as a bimolecular reaction, the reaction velocity of which is

$$\frac{da}{dt} = -Kab \quad (3)$$

where  $a$  and  $b$  represent the concentrations of the two reacting substances. The integration of equation 3 leads to

$$t = \frac{2.3}{K(a-b)} \log \frac{b(a-x)}{a(b-x)} \quad (4)$$

where  $a$  is the initial concentration of  $A$ , and  $b$  that of  $B$ , and  $x$  is the amount of substance decomposed in time  $t$ . If the reaction follows the kinetics of a bimolecular reaction (second-order reaction), the plot of  $\log (a-x)/(b-x)$  against  $t$  will yield a straight line. If  $a$  is equal to  $b$  or if two molecules of the same substance combine, then

$$\frac{1}{A} = Kt + D \quad (5)$$

where  $A$  is the concentration of  $A$  at any time  $t$ , and  $D$  is the integration constant. Under these circumstances the plot of  $1/A$  against  $t$  will yield a straight line. It must be pointed out, however, that the plot of the reciprocal of the concentration of a molecular species against time is not a general test for second-order reactions. It is a proper test only if the given molecular species is reacting with itself or if the initial concentrations of two molecular species are equal.

Examination of equations 4 and 5 shows that, in second-order reactions, the value of  $K$  is not independent of the manner in which the concentrations are expressed.  $K$  for such reactions has the dimensions of  $L^3/t$  and is usually expressed in liters per mole per second.

Enzymatic reactions as well as surface reactions frequently exhibit, over at least a part of their course, the kinetics of what is known as a zero-order reaction. In such a reaction the velocity is independent of the time and of the concentration, and

$$\text{Velocity} = -K \quad (6)$$

This behavior is usually due to a saturation of the surface (or enzyme) at which the reaction is taking place by the reactants; the further increase of the concentration of the reactants cannot increase the number of molecules which are active in the chemical reaction, and hence the velocity is independent of concentration.

It is frequently desirable to draw the best straight line through a series of experimental points; this is a general problem and by no means limited to kinetic studies. For this purpose the method of least squares is used. Consider the equation

$$y = a + bx \quad (7)$$

The constants  $a$  and  $b$  must satisfy the following criterion: the difference between the observed and the calculated results must be the smallest possible with small positive and negative differences. One of the best ways of fixing the numerical values of the constants is to make the sum of the squares of the differences between the observed and the calculated results as small as possible. It can be shown that this condition is true when the two constants in equation 7 have the following values

$$b = \frac{\Sigma(x)\Sigma(y) - N\Sigma(xy)}{[\Sigma(x)]^2 - N\Sigma(x^2)} \quad (8)$$

and

$$a = \frac{\Sigma(x)\Sigma(xy) - \Sigma(x^2)\Sigma(y)}{[\Sigma(x)]^2 - N\Sigma(x^2)} \quad (9)$$

where  $N$  is the number of experimental points and the symbol  $\Sigma$  means that all the values of the indicated functions are to be added together.

It is important to realize that the magnitude of the constants  $a$  and  $b$  depend on the selection of the dependent and of the independent variable. It is assumed that there is no error in the independent variable and all of the experimental deviations occur in the dependent variable. As equation 7 is written,  $x$  is always the independent variable and  $y$  is the dependent variable. It is also desirable to evaluate the error associated with the constants  $a$  and  $b$ . It is evident that, if  $y$  is plotted against  $x$ ,  $a$  is the intercept of the straight line and  $b$  is the slope of the line. The probable errors in  $a$  and  $b$  are

$$P_a = 0.6745 \sqrt{\frac{\Sigma d^2}{N-2}} \sqrt{\frac{\Sigma x^2}{N\Sigma x^2 - (\Sigma x)^2}} \quad (10)$$

$$P_b = 0.6745 \sqrt{\frac{\Sigma d^2}{N-2}} \sqrt{\frac{N}{N\Sigma x^2 - (\Sigma x)^2}} \quad (11)$$

where  $d$  is equal to the difference between the observed and the calculated values of  $y$ .  $x$  is the experimental value of this variable, and  $N$  is the total number of experimental observations.

As suggested before, the formulation of the kinetics of a reaction is not always practical, and we wish, therefore, to examine the two other ways mentioned above for expressing reaction velocities.



Some workers take the amount of reactant changed in a given time during the first part of the reaction as a measure of reaction velocity. Provided that the time interval is short, i.e., the amount of decomposition is small, this method is acceptable. In a first-order reaction or a bimolecular reaction which involves two different molecules whose initial concentrations are unequal, the ratio of the two velocity constants under different experimental conditions is equal not to the ratio of the two concentrations after a given time but to the ratio of a logarithmic function of their concentrations. If, therefore, the extent of reaction is appreciable, a comparison of the velocities in these cases and by this method will lead to a significant error.

The third method which has been used as a measure of reaction velocity is the determination of the reciprocal of the time required to effect a given amount of chemical change. If the amount of reactant changed is kept constant, then, no matter how complicated the reaction kinetics may be, we will have

$$K = \frac{M}{t} \quad (12)$$

where  $M$  is the term involving the concentration changes and which is by definition constant. Therefore, under different experimental conditions

$$\frac{K_2}{K_1} = \frac{1/t_2}{1/t_1} \quad (13)$$

where  $K_1$  and  $K_2$  are the velocity constants of a reaction under two different conditions and  $t_1$  and  $t_2$  are the times required to bring about a given chemical change. The reciprocals of the times required for a given change are, therefore, a true measure of the respective rates of reaction, and no knowledge of the reaction kinetics is necessary. The only assumption implicit in the argument is that, whatever the form of the true (but unknown) kinetic relation, it must be the same under the several conditions of the experiment; i.e., this method fails if the reaction changes its order from one condition to another.

The preceding discussion of reaction kinetics is a highly streamlined and simplified description. Many complicating factors have not been dealt with, such as opposing reaction, side reactions, and consecutive reactions. To enter into an exposition of all such complications would involve more time and space than we have at our disposal. It is perhaps fair to say that reaction kinetics presents more pitfalls to the unwary than any other branch of physical chemistry.

Having shown how the velocity of a reaction might be expected to vary with concentration of the reactants and having outlined the methods of



measuring reaction velocity, we now wish to consider the effect of temperature on a chemical reaction.

### EFFECT OF TEMPERATURE

Increase of temperature generally increases the velocity of a chemical reaction, and the temperature variation is usually expressed in terms of what is known as the  $Q_{10}$ . The  $Q_{10}$  is the ratio of the speed of reaction at one temperature to that at a temperature  $10^\circ$  lower. As a rule, the  $Q_{10}$  is in the neighborhood of 2. It is, however, not universally true that increase of the temperature increases the speed of a reaction. For example, the speed of enzymatically catalyzed reactions will always decrease as the temperature is raised above a certain point. This decrease is due to the destruction of the enzyme at higher temperatures.

Arrhenius found that the velocity of a chemical reaction could be related to the temperature by the relation

$$\log K = -\frac{E}{2.3RT} + B \quad (14)$$

and between the limits of two temperatures  $T_1$  and  $T_2$

$$\log \frac{K_2}{K_1} = \frac{E}{2.3R} \frac{T_2 - T_1}{T_2 T_1} \quad (15)$$

where  $R$  is the gas constant expressed in calories per degree per mole,  $T_1$  and  $T_2$  are the absolute temperatures, and  $K_1$  and  $K_2$  are the velocity constants corresponding to these temperatures.

The constant  $E$ , called the energy of activation, represents the energy that a mole of the reactant in the initial state must acquire before it will react to form products.

A consideration of the energy of activation helps us to understand the rather remarkable observation that a  $10^\circ$  rise in temperature can double and sometimes more than double the velocity of the reaction. The number of molecules with an energy content greater than the energy of activation can be calculated from the integrated Maxwell-Boltzmann distribution equation, which is

$$\frac{n_1}{n_0} = e^{-E/RT} \quad (16)$$

where  $n_1$  is the number of molecules with an energy in excess of  $E$ , and  $n_0$  is the total number of reactant molecules present. If the energy of activation is assumed to be 12,000 calories and the temperature to be  $300^\circ$  K. under these conditions the velocity of the reaction would be exactly dou-

bled in going from 26.8° C. to 36.8° C.) and we substitute these data in equation 16, we have

$$\frac{n_1}{n_0} = e^{-12,000/(2 \times 300)} = 2.06 \times 10^{-9}$$

while at 310° K.,

$$\frac{n_1}{n_0} = e^{-12,000/(2 \times 310)} = 3.93 \times 10^{-9}$$

Thus, whereas the average kinetic energy of the molecules has been increased only 3 per cent ( $\frac{10}{300}$ ) by the 10° increase in temperature, the fraction of molecules with energy greater than 12,000 calories has practically doubled.

### COLLISION THEORY AND ACTIVATION

On the basis of the collision theory of chemical reactions, the absolute velocity of a chemical reaction is given by the number of molecules reacting per second, which, in turn, equals the number of molecules colliding per second multiplied by the chance that the colliding particles have sufficient energy to react.

The Arrhenius equation 14 may be expressed in the following form:

$$\ln K = -\frac{E}{RT} + \ln Z \quad (17)$$

where  $\ln Z$  is equal to the integration constant  $B$ . Rearranging equation 17 we have

$$\ln \frac{K}{Z} = -\frac{E}{RT} \quad (18)$$

and converting this equation to the exponential form

$$K = Ze^{-E/RT} \quad (19)$$

From what has been said,  $e^{-E/RT}$  is evidently the chance that the colliding particles will have sufficient energy for a reaction to occur ( $e^{-E/RT}$  is the ratio of the activated molecules to the total number of reactant molecules present),  $Z$  is then the number of molecules colliding per second in unit volume, and  $K$  is now the actual number of molecules reacting per second per unit volume. In a monomolecular reaction,  $Z$  is to be regarded as the frequency of vibration of the activated bonds in the reacting molecules and is approximately the same for all monomolecular reactions (of the order of  $10^{13}$ ).

If the reactants are gases, then the collision rate can be calculated by means of the gas collision formula. However, if the reacting molecules are in solution, the mean free path of the molecules between collisions with each other is not the same as it is in a gas due to the Brownian motion of the reactant molecules and which arises from collision of the reactant molecules with the solvent molecules. Smoluchowski<sup>1</sup> in his investigations on the rate of precipitation of colloidal particles derived the following equation for the number of single particles in a suspension at any time  $t$

$$n = \frac{n_0}{1 + 8\pi r D n_0 t} \quad (20)$$

where  $n_0$  is the original number of particles,  $r$  is the radius of the particles,  $D$  is their diffusion constant, and  $t$  is the time. From equation 20 the rate of disappearance of single particles is evidently

$$\frac{dn}{dt} = - \frac{8\pi r D n_0 n}{1 + 8\pi r D n_0 t} \quad (21)$$

and at  $t$  equal to zero

$$\frac{dn}{dt} = -8\pi r D (n_0)^2 \quad (22)$$

Evidently, the collision rate is equal to one-half the rate of disappearance of single particles, and  $Z$ , the number of collisions per second, is  $4\pi r D (n_0)^2$ . If the two reacting particles have different radii and different diffusion constants, average values are used. The Smoluchowski derivation has been examined by Collins and Kimball<sup>2</sup> and its limitations pointed out. The collision rate calculated by means of the Smoluchowski equation is significantly less than the collision rate calculated from the gas collision formula.

It is possible to compare the collision rate as calculated by the Smoluchowski equation with the rate calculated from the energy of activation and the rate constant (equation 19). However, the rate of most reactions is not controlled by the collision rate except in an indirect manner, and the agreement between the two rates so calculated is usually very poor. To account for this lack of agreement equation 19 is written in the form

$$K = P Z e^{-E/RT} \quad (23)$$

where  $P$  is the so-called steric factor which may have any value from unity to zero, depending on the particular reaction under consideration.

<sup>1</sup> M. von Smoluchowski. *Physik. Z.* 27, 557, 583 (1916).

<sup>2</sup> F. C. Collins and G. E. Kimball, *J. Colloid Sci.* 4, 425 (1949).



## TRANSITION-STATE THEORY

There is, beside the collision theory outlined above, another approach to the whole problem of kinetics which involves a consideration of an activated complex. Eyring and Stearn<sup>3</sup> and collaborators have been most active along this line. The two methods of approach do not appear mutually exclusive; both have their advantages. The collision theory has its grounding in ideas that are simple and familiar to most scientific workers, and it involves calculations that are easy to make. The transition-state theory, on the other hand, involves basic ideas that are foreign to the experience of most biochemists, although the general picture which is presented is not a difficult one to grasp. One advantage of this type of approach is that all kinds of reactions are dealt with in a unified manner, whereas, on the basis of the collision theory, bimolecular and monomolecular reactions are treated differently.

The picture which Eyring and co-workers have given for reaction kinetics is about as follows: Before molecules can react they must pass through a configuration known as the activated state which has an energy content greater than that of the normal reactants. The average of this energy increment is the activation energy. The plot of the potential energy for various configurations shows that this activated state corresponds to a saddle point or pass between two hills. The pass leads to the decomposition of the activated configuration or, as it is called, the activated complex.

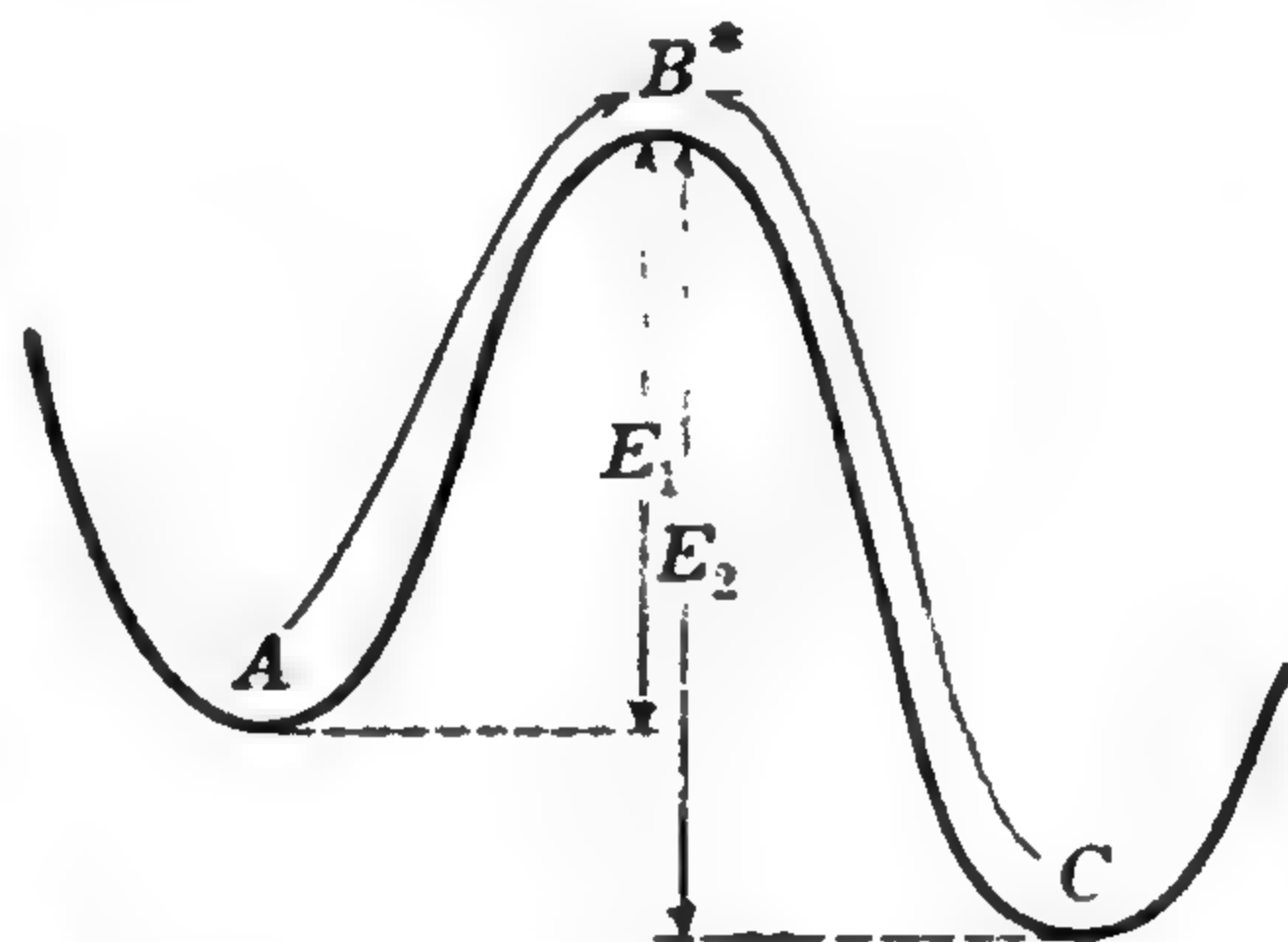


FIG. 1. Energy pass of a reaction.

Figure 1 shows a diagrammatic representation of a cross section of such a pass  $B^*$  leading from one energy valley  $A$ , which represents the normal state of the reactants, into a second energy valley  $C$ , which is the energy level of the products of the reaction. In Fig. 1  $E_1$  represents the free energy of activation of the forward reaction and  $E_2$  that of the opposing reaction.

The molecules pass from the normal state  $A$  to the activated state  $B^*$  and back again to the normal state  $A$ . Therefore, between the normal reactants and the activated complex, an equilibrium is established, which, however, is being continually disturbed by the activated complex "spilling" over into  $C$ . The problem resolves itself into the calculation of the concentration of the activated complex and the rate at which this complex

<sup>3</sup> A. E. Stearn, *Ergeb. Ensymforsch.* VII, 1 (1938).

H. Eyring and A. E. Stearn, *Chem. Revs.* 24, 253 (1939).

passes over the energy hump. The mean velocity of crossing the barrier is related to the energy of the activated molecules, and the concentration of the activated complex is obtained from wave mechanics. When necessary mathematical deductions are made it is found that the specific reaction velocity is

$$K = \frac{\gamma kT}{h} K^* \quad (24)$$

where  $K^*$  is the equilibrium constant between the normal reactants and the activated complex:  $kT/h$  is a frequency,  $k$  is the Boltzmann constant and is equal to  $1.37 \times 10^{-16}$  erg per degree,  $h$  is Planck's constant, equal to  $6.54 \times 10^{-27}$  erg-second,  $T$  is the absolute temperature.  $\gamma$  is the transition coefficient and represents the probability of the system in state  $B^*$  either returning to the normal state  $A$  or progressing to state  $C$ . The transition coefficient is a complicated function but for many reactions approaches the value of unity.  $K^*$ , the equilibrium constant, is related to the heat change, free energy change, and entropy change of activation in much the same manner as the equilibrium constant of any reaction is related to these thermodynamic quantities. We have, for example,

$$RT \ln K^* = T \Delta S^* - \Delta H^* = -\Delta F^* \quad (25)$$

and, accordingly,

$$RT \ln \frac{Kk}{kT} = T \Delta S^* - \Delta H^* = -\Delta F^* \quad (26)$$

$\Delta S^*$ ,  $\Delta F^*$ , and  $\Delta H^*$  are the standard entropy, standard free energy, and heat change for the reaction in which the activated complex is formed from the reactants.

It turns out that  $\Delta H^*$  is related to the energy of activation  $E$  as calculated by Arrhenius's equation in the following way

$$\Delta H^* = E - RT \quad (27)$$

It is evident that, for the usual values of the energy of activation and at lower temperatures (physiological temperatures), the energy of activation of Arrhenius is practically equal to the heat change of the activation reaction.

It is interesting to compare the collision treatment of the energy of activation with that of the transition-state treatment. In the exponential form equation 26 can be written

$$K = \frac{kT}{h} e^{\Delta S^*/R} \times e^{-\Delta H^*/RT} \quad (28)$$

At lower temperatures and appreciable energies of activation  $\Delta H^*$  and  $E$  are practically equal and we can write equation 28 (approximately) as

$$K = \frac{kT}{h} e^{\Delta S^*/R} e^{-E/RT} \quad (29)$$

Comparing equation 23 with equation 29, we see that

$$\frac{kT}{h} e^{\Delta S^*/R} = PZ \quad (30)$$

since  $Z$  and  $kT/h$  have the dimension of frequency and  $e^{\Delta S^*/R}$  and  $P$  are dimensionless,

$$\frac{kT}{h} = Z \quad (31)$$

and

$$P = e^{\Delta S^*/R} \quad (32)$$

The so-called steric factors of the collision theory are, therefore, related to the entropy change of the activation reaction.

#### INFLUENCE OF PRESSURE

At constant temperature but variable pressure, we have the relation

$$\Delta F^* = \Delta E^* + P \Delta V^* - T \Delta S^* \quad (33)$$

where  $\Delta E^*$  is the change in the internal energy,  $P$  is the pressure, and  $\Delta V^*$  is the difference in molar volume between the normal reactants and the activated complex. The rate equation becomes

$$K = \left( \frac{kT}{h} \right) e^{-\Delta E^*/RT} e^{-P \Delta V^*/RT} e^{\Delta S^*/R} \quad (34)$$

from which is obtained

$$\Delta V^* = -RT \left( \frac{d \ln K'}{dP} \right)_T \quad (35)$$

If the logarithm of the velocity constant be plotted against the pressure exerted on the system at constant temperature, the volume of the activated complex can be calculated from the slope of the line.

Johnson and co-workers<sup>4</sup> have studied the influence of pressure on a number of biological reactions and have calculated  $\Delta V^*$  for these reactions. Their apparatus is remarkably simple.

<sup>4</sup> F. H. Johnson et al., *J. Am. Chem. Soc.* 6, 725 (1946); *J. Cellular Comp. Physiol.* 26 43 (1945); *J. Biol. Chem.* 163, 689 (1946); *Proc. Natl. Acad. Sci.* 32, 21 (1946).



## REACTION KINETICS

There are certainly many advantages to the transition-state theory whether the energies of activation or the volumes of activation are calculated: it provides a logical approach to chemical kinetics. A difficulty arises, however, in the interpretation of the calculated values, particularly of the heat and entropy of activation. The free energy of activation of a given reaction must lie between fairly narrow limits in order to be subject to experimental measurements. If it is too small, the reaction is too rapid, and, if it is too large, the reaction rate is too slow and most reactions whose rate is capable of measurement will be in the neighborhood of 20,000 calories.

The heat of activation is interpreted in terms of the number of chemical bonds activated in the formation of the activation complex. Unfortunately, with a complex molecule no one knows what energies to assign to the individual bonds. For simple molecules the lengths of the bonds are increased about 10 per cent of their normal values, and the energy of activation is approximately one-fourth of the sum of the energies of the bond energies. The entropy of activation is related to the degree of randomness of the activated molecule as compared with that of the molecule in its normal state. However, with complex molecules such as proteins it is quite impossible to assign a quantitative interpretation to the entropy of activation. At first sight, the calculation of energies of activation for various biological reactions is impressive, but actually the amount of useful information that can be extracted from such studies is usually disappointing.

From equation 25 we see that the velocity of a reaction is dependent on the free energy of activation and that neither the heat of activation nor the entropy of activation alone will give a fair measure of the velocity of a reaction. It is, however, true that usually the contribution of the entropy of activation to the free energy of activation is distinctly smaller than is the heat of activation and, accordingly, the rate of reaction under these circumstances bears a relation to the heat of activation.

TABLE 1

ENERGIES OF ACTIVATION OF SOME CATALYZED AND NON-CATALYZED REACTIONS  
(Lineweaver)

Reaction	Catalyst	<i>E</i>
H <sub>2</sub> O <sub>2</sub> decomposition	None	18,000
	Colloidal platinum	11,700
	Liver catalase	5,500
Sucrose inversion	H ions	26,000
	Yeast invertase	11,500
Casein hydrolysis	HCl	20,600
	Trypsin	12,000
Ethyl butyrate	H ions	13,200
	Pancreatic lipase	4,200

The general function of enzymes is to reduce the energy of activation for a given reaction. The enzyme thus "tunnels" under the energy pass between the reactants and the resultants of the reaction. In general, therefore, enzymatic reactions have a lower temperature coefficient than corresponding non-enzymatic reactions. Lineweaver<sup>5</sup> gives the energies of activation of some of the non-catalyzed and catalyzed reactions. (See Table 1.)

### $Q_{10}$ IN BIOLOGY

The activity of life is greatly influenced by the temperature of the environment; in general, life processes increase their speed with increasing temperature. Attempts have been made to analyze this temperature dependence in terms of the Arrhenius equation 14. It is believed by Crozier and his associates that the speed of a given process (such as the rate of beat of insects' wings, for example) is controlled by a master reaction (slowest reaction) and that the change in physiological activity with temperature is a reflection of the change in the velocity of the master reaction. Not infrequently the energy of activation of a living process is not constant but varies with temperature. The explanation advanced by Crozier is that the living process is made up of a series of reactions and as the temperature is changed a different reaction of the series becomes the slowest, which results in an altered energy of activation.

Crozier has found that the energies of activation for life processes have a tendency to cluster about certain values, i.e., 8,000, 12,000, and 18,000.

The interpretation of Crozier has been severely criticized by certain workers. For example, Ponder and Yeager state that (1) "If there are several reactions involved in bringing about the total response, it is mathematically demonstrable that the slope of the line (or curve) is not determined by the temperature coefficient of any one of them alone; an activation value of 10,000, say, derived from this line, does not indicate that any one of the individual reactions has this temperature coefficient, or even that the average energy of activation of the reaction is 10,000. (2) The fact that the entire system appears to follow the Arrhenius equation does not even necessarily indicate that any one of the underlying reactions does so; further, if a series of values are obtained for various parts of the temperature range, there is no reason to suppose (a) that each new slope corresponds to a new reaction or (b) that the values obtained for various slopes have any direct relation to the temperature coefficients of the various underlying reactions in the system under consideration."

Morales<sup>6</sup> has, however, pointed out that an enzyme system in a steady

<sup>5</sup> H. Lineweaver, *J. Am. Chem. Soc.* 61, 403 (1939).

<sup>6</sup> M. F. Morales, *J. Cellular Comp. Physiol.* 30, 303 (1947).



state and involving a series of consecutive reactions can be interpreted in terms of a reaction in which the enzyme-substrate complex is split and in terms of another reaction in which the enzyme is reversibly inactivated. A plot of the logarithm of the velocity of the reaction against the reciprocal of the temperature under such circumstances will always give a curve which is concave downwards. At the lower temperatures the entire reaction is controlled by the rate at which the enzyme-substrate complexes are split. At higher temperatures both this reaction and the reversible inactivation equilibrium are governing the overall rate. By properly taking into account the temperature dependence of these two reactions in which the enzyme is involved, it is possible to obtain from the rate data thermodynamic information about both.

Kistiakowsky and Lumry<sup>7</sup> have investigated the influence of temperature on the rate of hydrolysis of urea by urease in the presence as well as absence of sodium sulfite. They find that the energy of activation for the hydrolysis of urea by urease in the absence of sulfite is over the temperature range 5° to 20° C. constant and is 8,850 calories. However, in the presence of sulfite the energy of activation increases as the temperature is lowered reaching values of around 14,000 calories, whereas at higher temperatures (24° to 30° C.) the energy of activation in the presence of sulfite approaches that in the absence of sulfite, i.e., 8,800 calories. They believe the change of activation energy is a reflection of the reversible inactivation of urease by sulfite. Their argument, therefore, is along the lines set forth by Morales and noted above.

### ENZYMATIC ACTIVATION

Enzymes are a group of organic compounds of a highly specific nature whose function is to direct biochemical reactions into useful channels. A number of enzymes have been isolated and purified to a sufficient extent to obtain crystals. All enzymes so far prepared in a pure state are proteins.

Almost all reactions occurring in living systems are directed by enzymes, and, accordingly, there are a very large number of enzymes the characters of which are diverse. A group of these enzymes is concerned with oxidation-reduction reactions and with electron transfer. Another group catalyzes the hydrolysis of various substances: its primary function appears to be proton transfer. Enzymes, in general, show a high degree of specificity although Neurath et al.<sup>8</sup> have been able to show that trypsin, carboxy-

<sup>7</sup> G. B. Kistiakowsky and R. Lumry, *J. Am. Chem. Soc.* 71, 2006 (1949).

<sup>8</sup> G. W. Schwert, H. Neurath, S. Kaufman, and J. E. Snoke, *J. Biol. Chem.* 172, 221 (1948).

J. E. Snoke, G. W. Schwert, and H. Neurath, *J. Biol. Chem.* 175, 7 (1948).

S. Kaufman, G. W. Schwert, and H. Neurath, *Arch. Biochem.* 17, 203 (1948).



peptidase, and chymotrypsin are capable of hydrolyzing esters as well as peptides. The three enzymes, however, require the same specific environment of groups about the bond that they hydrolyze, whether it be a peptide or an ester bond although different for each enzyme.

In common with all true catalysts, enzymes do not and cannot influence the equilibrium point of a reaction; they serve only to decrease the time required to attain equilibrium and to "lubricate" the reaction.

Since an enzyme increases the speed of reaction in the forward direction without contributing energy to the system, it follows that the speed of the reverse reaction should also be increased. This conclusion is clear from the simple relation

$$\frac{K_f}{K_r} = K_e \quad (36)$$

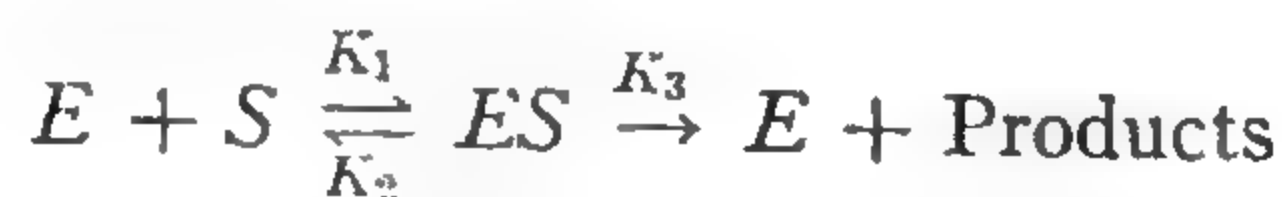
where  $K_f$  and  $K_r$  are the velocity constants in the forward and in the reverse direction, respectively, and  $K_e$  is the equilibrium constant of the reaction which is unchanged by the enzyme. This concept, however, needs some qualification. It is true that an enzyme does not change the total energy of a system, but no one in his right scientific mind believes that pepsin placed in a peptic digest of a protein along with a dehydrating agent so that the free energy is in the direction of synthesis will ever succeed in synthesizing the original protein. During the hydrolysis of the protein there has been a very great increase of entropy which makes it very improbable that the original protein could be synthesized by pepsin. Pepsin would no doubt facilitate the synthesis of some peptide bonds, but these bonds would not necessarily be the same as those originally present in the protein. On the other hand, the resynthesis of an ester from a monohydroxy alcohol and a fatty acid permits of only one choice of combination and considerable success has been attained in the synthesis of such compounds by esterases.<sup>9</sup>

## ENZYME KINETICS

The kinetics of enzymatic reactions are frequently complicated, and the order of the reaction may change during the course of the reaction. At the beginning of the reaction and at high substrate concentration the reaction may exhibit the kinetics of a zero-order reaction; this stage corresponds to a saturation of the enzyme surface by the substrate. As the reaction proceeds and the substrate concentration decreases, the reaction usually reverts to the kinetics of a first-order reaction. It cannot be too strongly urged that, in comparing the influence of various factors on the velocity of enzymatic reactions, a proper measure of the velocity be chosen.

<sup>9</sup> E. A. Sym, *Biochem. J.* 24, 1265 (1930).

The point of departure for a formulation of enzyme kinetics is the Michaelis-Menten treatment.<sup>10</sup> The foundation of their analysis is the assumption that the enzyme and the substrate form an intermediate complex which dissociates into free enzyme and products of the reaction, as well as giving rise to free substrate,



If  $E_0$  represents the total concentration of enzyme,  $E$  the concentration of free enzyme, and  $ES$  the concentration of bound enzyme, then

$$E = E_0 - ES \quad (37)$$

The dissociation constant of the enzyme-substrate complex is

$$K_m = \frac{E \times S}{ES} = \frac{(E_0 - ES)S}{ES} \quad (38)$$

or by rearrangement

$$ES = \frac{E_0 \times S}{K_m + S} \quad (39)$$

The velocity of the reaction for the formation of the end products is

$$v = K_3 \times ES \quad (40)$$

where  $K_3$  is the velocity constant for the break up of  $ES$  to form free enzyme and products. Then substitution of equation 40 into equation 39 gives

$$v = \frac{K_3 \times E_0 \times S}{K_m + S} \quad (41)$$

Now, if the substrate concentration be increased to the point where the surface of the enzyme is saturated with the substrate, then the maximum velocity of the reaction is

$$V = K_3 \times E_0 \quad (42)$$

or substituting equation 42 in equation 41 gives

$$v = \frac{V \times S}{K_m + S} \quad (43)$$

The constants of equation 43 can be conveniently evaluated by taking the reciprocals of both sides to give

$$\frac{1}{v} = \frac{K_m}{V \times S} + \frac{1}{V} \quad (44)$$

<sup>10</sup> L. Michaelis and M. L. Menten, *Biochem. Z.* **49**, 333 (1913).

Evidently, a plot of  $1/r$  against  $1/S$  should yield a straight line the slope of which is  $K_m/V$  and the intercept is  $1/V$ . A somewhat more critical method for the evaluation of constants is to multiply equation 44 by  $S$  giving

$$\frac{S}{r} = \frac{K_m}{V} + \frac{S}{V} \quad (45)$$

$S/r$  is then plotted against  $S$ . The constants are then evaluated from the slope and the intercept of the straight line.

The integration of equation 43 yields

$$S - S_0 + K_m \ln \frac{S}{S_0} = Vt \quad (46)$$

which is composite zero and first-order reaction. The zero-order will predominate when  $S$  is large and  $K_m$  is small. The first order will predominate when  $S$  is small and  $K_m$  is large.

It is astonishing how generally applicable the Michaelis-Menten formulation turns out to be, and it is a tribute to the simplicity and genius of their derivation. It must be realized, however, that  $K_m$  is not a dissociation constant in the usual sense of the term. The rate of formation of  $ES$  is

$$\frac{dES}{dt} = K_1(E_0 - ES)S \quad (47)$$

and the rate of decomposition of  $ES$  is

$$\frac{dES}{dt} = -(K_2 \times ES + K_3 \times ES) \quad (48)$$

If the rate of the overall reaction is constant, then  $ES$  must be constant and

$$K_1(E_0 - ES)S = K_2 \times ES + K_3 \times ES \quad (49)$$

and solving equation 49 for  $ES$ , we have

$$ES = \frac{S \times E_0}{S + \frac{K_2 + K_3}{K_1}} \quad (50)$$

Comparing equation 50 with equation 39 we see that  $K_m$  is really equal to  $(K_2 + K_3)/K_1$ , and it is only when  $K_3$  is very much smaller than  $K_2$  that  $K_m$  becomes equal to the true dissociation constant of the complex.

It can be shown<sup>11</sup> that the Michaelis-Menten substrate-enzyme complex

<sup>11</sup> H. B. Bull and B. T. Currie, *J. Am. Chem. Soc.* 71, 2758 (1949).



is not the activated complex: the concentration of the activated complex is far smaller than that of the Michaelis-Menten complex. This conclusion means that the Michaelis-Menten complex must pass over into an activated complex before it can decompose into the final products of the reaction. This represents an additional complication which must be taken into account.

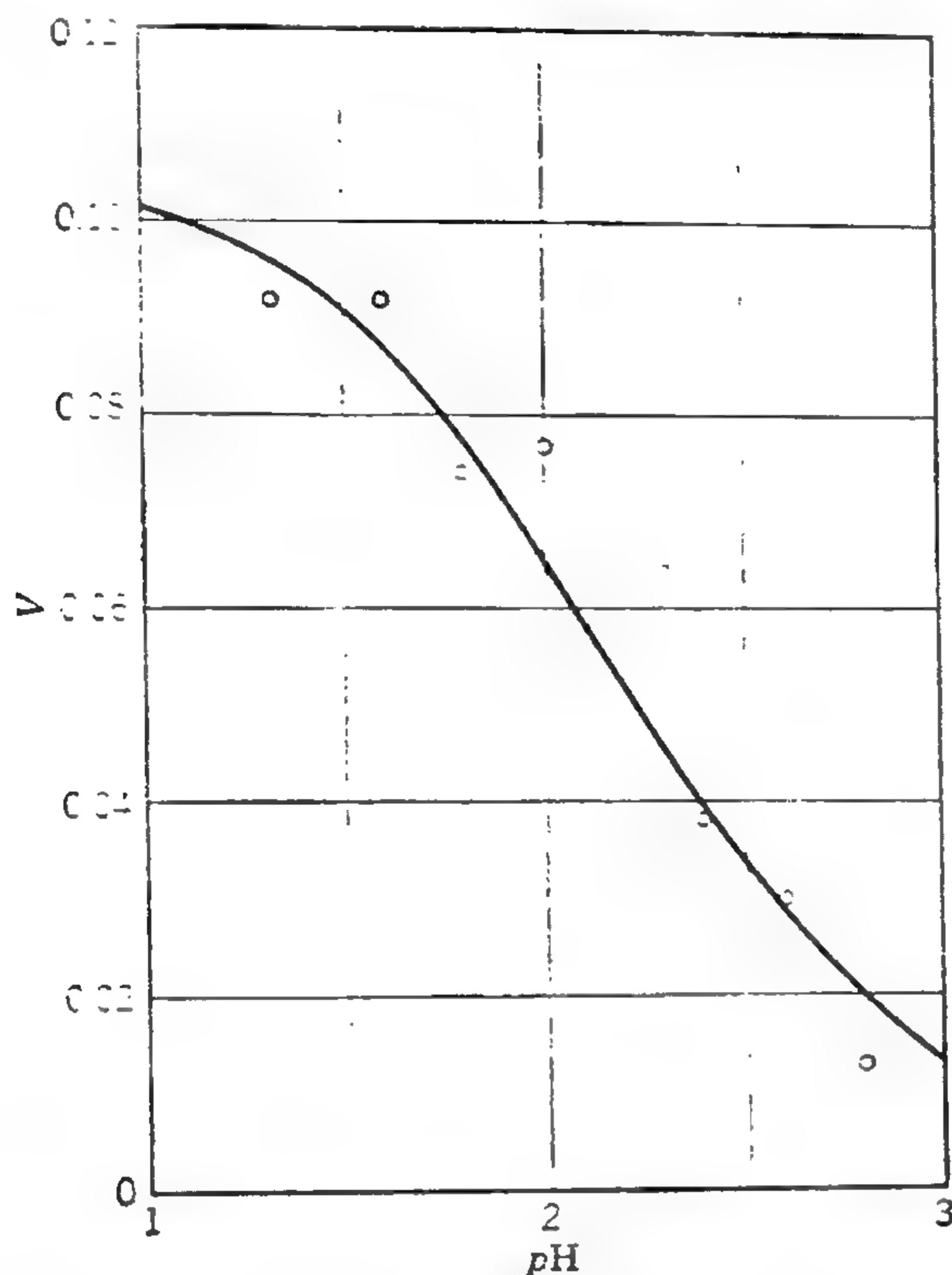


FIG. 2. Plot of experimental points for the maximum velocity of digestion of egg albumin against pH for  $50^{\circ}$ . The solid line has been calculated from theory.

Hydrogen ions are frequently of great importance in enzyme reactions and the reaction rate is a marked function of the hydrogen-ion concentration. This dependence can arise from either binding of protons by the substrate, by the enzyme, or by the enzyme-substrate complex or by a combination of these. Figure 2 shows the variation of the maximum rate of reaction  $V$  of the digestion of egg albumin by pepsin. It is considered that the rate curve shown in Fig. 2 is in effect an acid titration curve of the enzyme-substrate complex and that the sequence of reactions leading to digestion of egg albumin by pepsin can be written



where  $E$  represents the pepsin,  $S$  the egg albumin, and  $P$  the split products

of the reaction. Figure 3 shows a heat of activation profile of the reactions given above. It can be seen that the sequence of energy changes leading to digestion is complex and it is quite misleading to represent them by a single energy of activation.

The results of Chance<sup>12</sup> are very impressive because he actually demonstrated the separate existence of the substrate-enzyme complex. He concerned himself, along with other enzymes, with the peroxidase from horse-

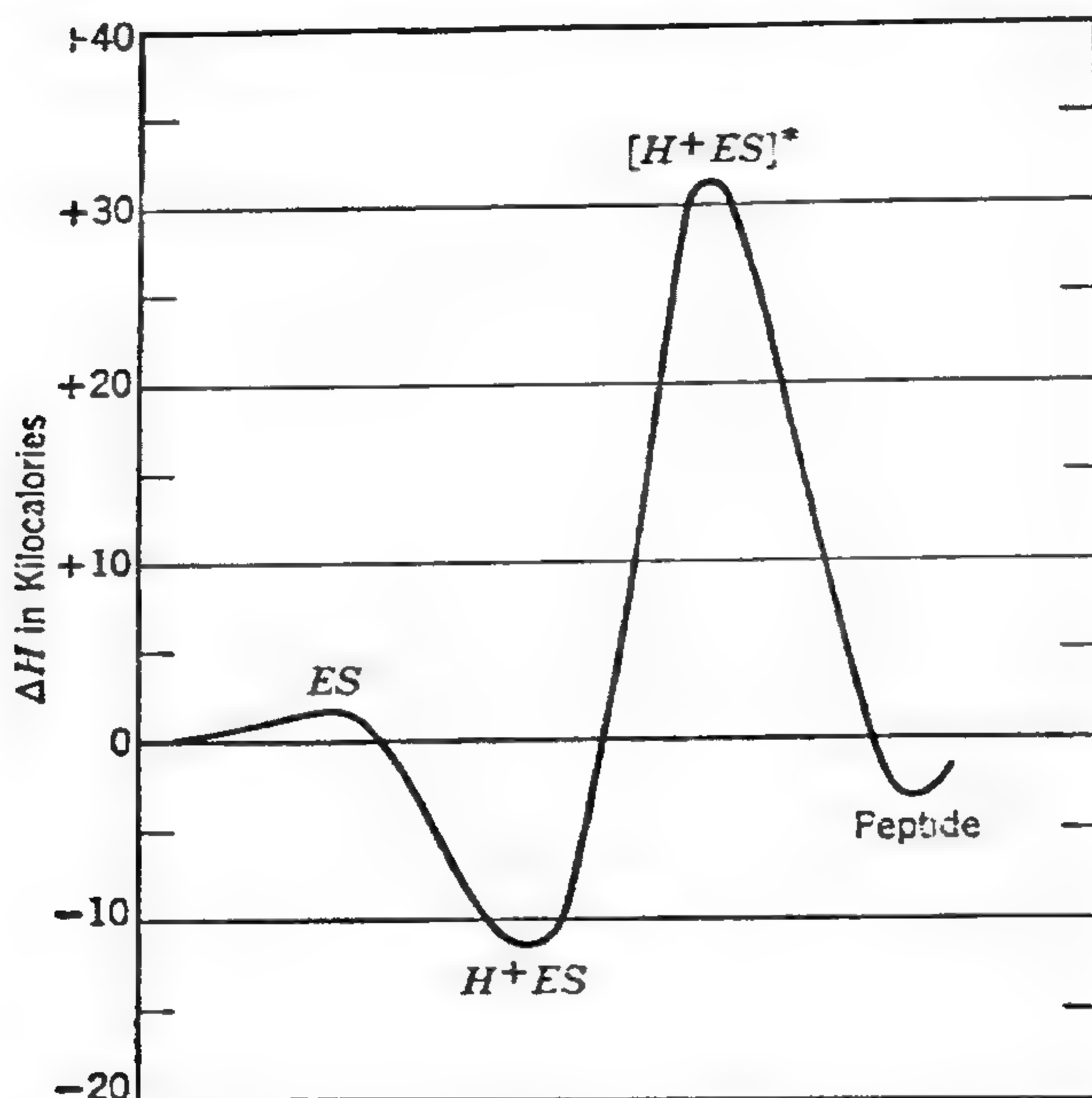


FIG. 3.  $\Delta H$  profile for the reactions leading to the digestion of egg albumin by pepsin.

radish. This enzyme has the property of combining with hydrogen peroxide forming a very unstable complex which is green. In this complex, the iron of the peroxidase probably form ionic bonds. This complex passes rapidly over into a red complex. During this transition the ionic bonds of the iron probably revert to covalent bonds. The complex then combines with the appropriate oxygen acceptor such as ascorbic acid and liberates the free enzyme. By using a rapid flow apparatus, it was possible to study the transit color changes as these colors proceeded from the brown of the free enzyme, to the green complex, to the red complex, and finally to the reaction products with the regeneration of the brown enzyme. The reversible decomposition of the enzyme-substrate complex does not appear to play an important role in the reaction kinetics; in fact, final proof of the

<sup>12</sup> B. Chance, *Nature* 161, 914 (1948); *Sci.* 109, 204 (1949); *Arch. Biochem.* 21, 416 (1949); 22, 224 (1949).

existence for a finite value of the dissociation constant is lacking. Hence the rapidly reversible combination of enzyme and substrate pictured in the Michaelis-Menten formulation is not characteristic of the peroxidase enzyme.

### INHIBITORS

Inhibitors of enzymatic reactions are traditionally divided into two types: competitive and non-competitive. In competitive inhibition, the inhibitor competes with the substrate for the specific groups of the enzyme and, accordingly, the velocity of the catalyzed reaction depends on the relative concentrations of the substrate and of the inhibitor. In non-competitive inhibition, the inhibitor inactivates the enzyme by combination with groups not involved in substrate binding and hence inactivation depends only on the concentration of the inhibitor.

The mathematical formulation of the action of inhibitors has been given by Lineweaver and Burk.<sup>13</sup> Consider the reactions



and



where  $I$  represents the inhibitor. We can represent both of these reactions by equilibrium constants

$$K_m = \frac{E \times S}{ES} \quad \text{and} \quad K_I = \frac{E \times I}{EI} \quad (51)$$

Combining these two expressions we have

$$EI = \frac{K_m \times ES \times I}{K_I \times S} \quad (52)$$

And since

$$E = E_0 - ES - EI \quad (53)$$

we have upon combining equation 52 with equation 53

$$E = \frac{K_m \times ES}{S} = E_0 - ES - \frac{K_m \times ES \times I}{K_I \times S} \quad (54)$$

And substituting equations 40 and 42 into equation 54 and rearranging, we have

$$v = \frac{V}{\frac{K_m}{S} + 1 + \frac{K_m \times I}{K_I \times S}} \quad (55)$$

<sup>13</sup> H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).



Taking the reciprocals of both sides of equation 55 and rearranging the results

$$\frac{1}{v} = \frac{K_m}{V \times S} \left( 1 + \frac{I}{K_I} \right) + \frac{1}{V} \quad (56)$$

when  $1/v$  is plotted against  $1/S$ , the slope of the line is  $K_m / V (1 + I/K_I)$  which represents an increase of the slope over the uninhibited slope of  $(1 + I/K_I)$ . The intercept, however, remains unchanged.

By the same type of approach as outlined above it can be shown that the rate reaction for non-competitive inhibition can be represented by

$$\frac{1}{v} = \left[ 1 + \frac{I}{K_I} \right] \left[ \frac{1}{V} + \left( \frac{K_m}{V} \right) \frac{1}{S} \right] \quad (57)$$

It is evident from equation 57 that if  $1/v$  be plotted against  $1/S$  both the slope and the intercept are increased by the factor  $(1 + I/K_I)$ . Competitive and non-competitive inhibition can thus be distinguished from each other. There are other and more complex types of inhibition.<sup>14</sup>

Neurath and Schwert<sup>15</sup> have written a splendid review of the proteolytic enzymes from the pancreas in which they deal with the problem of enzyme kinetics, specificity, and mode of action of these enzymes.

## CHAIN REACTIONS

In a chain reaction a molecule is activated by contact with the sides of a container, by an enzyme, by absorbed radiation, or by solvent molecules. This "hot" or activated molecule is able to activate another molecule by collision, after which it decomposes; the newly activated molecule is able to repeat this performance. This activating series forms what is known as a chain which may have several thousand links in it before it is broken. If the chains become branched and are not inhibited, an explosion results. A chain reaction bears the six following characteristics:<sup>16</sup>

1. At least one of the stages in the sequence of the reaction must be exothermic (give off heat); accordingly, it is able to activate other molecules.
2. The velocity of a chain reaction is a function of the number of active centers produced per unit time in unit volume, and of the average length of the chain. Therefore, there will be no simple relation between the concentration of the reactants and the instantaneous rate of reaction.
3. Velocity of the reaction may be profoundly altered by the presence of an added foreign substance which breaks the chain.

<sup>14</sup> P. W. Wilson, *Respiratory Enzymes*, edited by H. A. Lardy, Burgers Publishing Co. Minneapolis, 1949.

<sup>15</sup> H. Neurath and G. W. Schwert, *Chem. Revs.* **46**, 69 (1950).

<sup>16</sup> E. A. Moelwyn-Hughes, *Ergeb. Enzymforsch.* **VI**, 23 (1937).

## REACTION KINETICS

4. The reaction may involve an induction period during which the chains are forming.

5. If the chains are initiated or terminated on the walls of the vessel, the velocity of the reaction will depend on the size or shape or on the nature of the walls of the vessel.

6. The chains may branch, and unchecked acceleration leads to an explosion.

It is doubtful that living systems make use of such a poorly controlled process as a chain reaction: certainly the kinetics of reactions catalyzed by proteases, carbohydrases, and lipases do not suggest a chain reaction. It is possible that the oxidative enzymes, since they involve an exothermic step, may pass through a chain reaction, although to the author this seems improbable. It is true, however, that the catalase system and the oxidation of luciferin, catalyzed by luciferase, are adequately described by the kinetics of a chain mechanism.<sup>17</sup>

It seems very probable that a chain mechanism is involved in the drying of oils, as well as in oil rancidity.<sup>18</sup> Polymerization of the oil is also thought to be a chain reaction. In fact, the two reactions are probably closely connected. The activation energy received from oxidation may serve to activate molecules for the polymerization reaction. In rancid oils the chains can be broken by small amounts of anti-oxidants, hydroquinone being an example of such an anti-oxidant.

## PROBLEMS AND QUESTIONS

1. Egg albumin solutions were heated at 65° C. and at 70.2° C. The weights of soluble protein per cubic centimeter remaining at various time intervals were measured with the following results:

65° C.		70.2° C.	
Hours	Mg. Soluble Protein	Minutes	Mg. Soluble Protein
0	7.77	0	6.75
5	7.37	30	6.13
10	7.00	80	5.25
20	6.32	120	4.68
30	5.72	200	3.72
40	5.22	300	2.76

Calculate the velocity constant of the heat denaturation of egg albumin, the free energy the entropy, and the heat of activation per mole at 65° C.

Ans.:  $K = 2.8 \times 10^{-6}$ ;  $\Delta F^\circ = 28,700$ ;  $\Delta H^\circ = 125,300$ ;  $\Delta S^\circ = 286$

<sup>17</sup> E. N. Harvey, *J. Gen. Physiol.* 10, 875 (1927).

<sup>18</sup> I. A. Christiansen, *Trans. Faraday Soc.* 24, 714 (1928).

2. One milligram of crystalline pepsin was dissolved in 100 cc. of each of three solutions containing 0.486, 0.953, and 2.97 grams of egg albumin at 25° C. and at pH 2.0. The initial velocities of digestion for these three solutions were 0.117, 0.165 and 0.235 grams of protein per hour, respectively. The molecular weight of pepsin is 35,000 and that of egg albumin is 45,000. Calculate the maximum velocity of digestion in moles of egg albumin per second per mole of pepsin, as well as the Michaelis-Menten dissociation constant in molar quantities.

*Ans.:* 0.064 mole per second per mole;  $1.6 \times 10^{-4}$ .

3. Derive the expression for non-competitive enzymatic inhibition.



## Chapter

# 4

## IONS in SOLUTION

As a result of his studies on electrolysis, Faraday called those bodies that migrate to the anode anions and those that move to the cathode upon the passage of an electric current through a salt solution cations. The word "ion" is from the Greek meaning wanderer.

Arrhenius<sup>1</sup> considered that ions existed as such in solution and needed no electric current to produce them. He found that in a number of instances he could formulate the dissociation of the electrolyte in terms of an equilibrium between the dissociated and the undissociated parts of the dissolved molecule. For example, for acetic acid, we have



which can be expressed in the form of an equilibrium constant

$$K = \frac{\text{H}^+ \times \text{Ac}^-}{\text{HAc}} \quad (1)$$

In this early work the magnitude of  $K$  was determined by measuring the electrical conductivity of a series of dilutions of the electrolyte. Today there are less ambiguous methods for the evaluation of  $K$ ; these methods will be discussed in some detail in Chapter 7.

We, of our time, take the concept of ionization more or less for granted, but it can easily be imagined how revolutionary it must have seemed when it was proposed. The people of Arrhenius' day must have asked why these ionic charges did not neutralize each other; we should still ask that question.

There are, in general, two types of chemical bonds which hold atoms together (a third type, the metallic bond, will not be considered).<sup>2</sup> The first of these is the covalent bond, which results from the sharing of electrons by two atoms. Examples of this type are the carbon-carbon bond and the carbon-hydrogen bond. Covalent bonds do not permit dissociation of a

<sup>1</sup> S. Arrhenius, *Z. physik. Chem.* 1, 631 (1887).

<sup>2</sup> L. Pauling, *The Nature of the Chemical Bond*, Cornell University Press, 1940.

molecule into ions. The second type is the ionic bond (sometimes called a salt linkage). The ionic bond arises from an actual transfer of electrons from one atom to another. An illustration is found in ordinary sodium chloride. The metal atom, sodium, gives up an electron to the chlorine atom. This exchange leaves the sodium positively charged and imparts a negative charge to the chlorine, producing a sodium and a chloride ion, respectively. An electrostatic attraction between the sodium and chloride results. This is the ionic bond, and the attractive forces between the ions are known as coulombic forces.

The transfer of an electron from the sodium atom to a chlorine atom leaves the sodium with a completed electron shell, which is a rare-gas structure. The transfer completes the chlorine electron shell and gives it also a rare-gas structure. Such structures are very stable. In a crystal of sodium chloride, sodium and chlorine do not exist as atoms, but as ions; a similar statement is in general true of such crystals (KCl, NaBr, etc.).

The existence of ions in solution is conditioned by two factors: (1) the attraction of ions for each other, and (2) ionic hydration. The force between charged particles is given by

$$\text{Force} = \frac{Q_1 Q_2}{D d^2} \quad (2)$$

where  $Q_1$  and  $Q_2$  are the charges on the particles,  $d$  is the distance between the charges, and  $D$  is the dielectric constant. Water with a dielectric constant of 78.54 at 25° C. decreases the force between the ions to  $\frac{1}{78.54}$  of that existing in ionic crystals. Evidently this factor will enhance the solubility of ionic crystals in water. The second factor, ionic hydration, will presently be dealt with in detail. It is clear, however, that hydration will decrease the potential energy of an ion and will, accordingly, enhance the separation of ions. If the substance in question does not exist as ionic crystals but is a weak electrolyte, such as acetic acid, the two factors mentioned above are important in determining the extent of ionization of the compound. For example, decreasing the dielectric constant of water by the addition of dioxane decreases to a very marked extent the ionization of formic acid.<sup>3</sup>

## SIZES OF IONS

Table 1 gives the crystal radii of some ions. The crystal radius is defined as that distance such that the sum of the radii of any two ions is equal to the actual equilibrium interionic distance in a crystal containing the ions.

<sup>3</sup> H. S. Harned and R. S. Done, *J. Am. Chem. Soc.* 63, 2579 (1941).

## IONS IN SOLUTION

TABLE 1

## IONIC RADII

From *The Nature of the Chemical Bond*, by L. Pauling

Ion	Radius in Å	Ion	Radius in Å
Li <sup>+</sup>	0.60	Ba <sup>++</sup>	1.35
Na <sup>+</sup>	0.95	F <sup>-</sup>	1.36
K <sup>+</sup>	1.33	Cl <sup>-</sup>	1.81
Rb <sup>+</sup>	1.48	Br <sup>-</sup>	1.95
Cs <sup>+</sup>	1.69	I <sup>-</sup>	2.16
Mg <sup>++</sup>	0.65	SO <sub>4</sub> <sup>=</sup>	1.51
Ca <sup>++</sup>	0.99	NO <sub>3</sub> <sup>-</sup>	1.21
Sr <sup>++</sup>	1.13	SCN <sup>-</sup>	3.03

## INTERIONIC ATTRACTION

It became apparent rather early that Arrhenius' idea of partial ionization did not always satisfy quantitative formulation. This was particularly true of many salts and of such strong acids as hydrochloric and sulfuric. In these substances it was found that the calculated equilibrium constant of ionization was a pronounced function of the electrolyte concentration.

Some help for the treatment of such electrolytes (known in general as strong electrolytes) was forthcoming from the empirical discovery by Lewis and Randall <sup>4</sup> that the effect of ions in dilute solutions was proportional, not to their molar concentration, but to their ionic strengths, and they enumerated the important rule: "In dilute solutions, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength." They defined the ionic strength,  $\mu$ , as equal to the sum of the ionic concentrations, each ionic concentration being multiplied by the square of the ionic valence. The whole sum is divided by 2 (concentrations are expressed in gram ions per liter). That is

$$\mu = \frac{C_1Z_1^2 + C_2Z_2^2 + C_3Z_3^2}{2} \quad (3)$$

Consider 0.01 molar solutions of NaCl, MgSO<sub>4</sub>, and H<sub>2</sub>SO<sub>4</sub>. These solutions have the following ionic strengths:

$$\begin{aligned} \mu \text{ for NaCl} &= \frac{0.01 \times 1^2 + 0.01 \times 1^2}{2} = 0.01 \\ \mu \text{ for MgSO}_4 &= \frac{0.01 \times 2^2 + 0.01 \times 2^2}{2} = 0.04 \end{aligned}$$

<sup>4</sup> G. N. Lewis and M. Randall, *Thermodynamics*, McGraw-Hill Book Co., Inc., New York, 1923.



$$\mu \text{ for H}_2\text{SO}_4 = \frac{0.01 \times 1^2 + 0.01 \times 1^2 + 0.01 \times 2^2}{2} = 0.03$$

The rule given above, that solutions of all strong electrolytes at the same ionic strengths exhibit the same ionic effects, was found to be valid up to an ionic strength of about 0.1.

The principle of ionic strength is an important one for the biochemist. Frequently, for example, a study is made of the variation of some factor as a function of the  $pH$  of a solution, buffers being employed to yield the proper  $pH$ . In order, however, for the effect observed to be due only to the variation in  $pH$  and not obscured by secondary salt effects, it is necessary to make up the series of buffers to the same ionic strength. This does not always suffice, as many physiological responses have specific ion effects. Nevertheless, a constant ionic strength does represent the least that the biochemist can do to make his experiment as unambiguous as possible.

The failure of the Arrhenius formulation for salts, strong acids, and bases led several people to the idea that such electrolytes are at all times completely dissociated and that the anomalous effects were due to electrostatic interaction of the ions. Debye and Hückel<sup>5</sup> were the first to formulate this concept in a satisfactory mathematical manner. Their contribution to chemistry must be considered one of the most important in this century. We shall attempt to outline their approach:

Debye and Hückel made two assumptions: (1) that ionization of strong electrolytes was complete; and (2) that the deviation from ideal behavior was due to the electrostatic effects of the positive and negative ions on one another and to no other cause. Their derivation may conveniently be divided into three steps. First, they proceeded to derive an expression for the distribution of positive ions around a negative one.

Evidently, owing to the presence of a negative ion there will be an excess of positive ions in its immediate neighborhood. The difference in concentration between the negative and positive ions will determine the charge density in the neighborhood of a negative ion. If it is difficult for the reader to visualize such an excess of positive ions, consider the extreme example of a sodium chloride crystal. Here, a chloride ion is entirely surrounded by six sodium ions. The situation in solution naturally does not approach the regularity of a crystal.

The concentration of positive and negative ions in the neighborhood of a negative ion was calculated by means of the Boltzmann principle, which can be expressed mathematically as

$$n = n_0 e^{W/kT} \quad (4)$$

<sup>5</sup> P. Debye and E. Hückel, *Physik. Z.* 24, 185, 334 (1923).

where  $n_0$  is the number of ions per unit volume for a homogeneous distribution (no electrostatic effects);  $n$  is the number of ions per unit volume in the presence of the electrostatic field;  $W$  is the work required to take the ions from the homogeneous distribution into a place where the electrostatic field is acting;  $k$  is the Boltzmann distribution constant; and  $T$  is the absolute temperature. The sign of the exponent is positive for a positive ion and minus for a negative one. Combining the Boltzmann expression with the knowledge that the difference in concentration of the negative and positive ions is equal to the charge density, we find (after considerable mathematical manipulation which involves the expansion of the exponential Boltzmann equation and neglecting second-order terms) that the charge density is

$$\rho = - \frac{N^2 \epsilon^2 \Phi}{1,000 RT} [C_1 Z_1^2 + C_2 Z_2^2] \quad (5)$$

where  $N$  is Avogadro's number,  $\epsilon$  is the electronic charge,  $R$  is the gas constant,  $C_1$  and  $C_2$  are the concentration in gram ions per liter,  $Z_1$  and  $Z_2$  are the respective valences, and  $\Phi$  is the potential existing at the location of the ion.

The second step in the derivation was to use the differential form of Poisson's equation, which relates the charge density and the potential. The charge density as given by equation 5 was substituted in the Poisson equation and the equation was integrated over the entire ionic atmosphere around the negative ion to obtain the potential. This potential when multiplied by the charge on the ion represents the electrical work required to remove the ion from its environment in the solution. When this work is summed up for all the negative as well as for all the positive ions in solution, we have the total potential energy decrease suffered by the solution due to the electrostatic charges present. Carrying out this summation we find that the total work is

$$\text{Work} = \frac{N Z_1 Z_2 \epsilon^2 \kappa}{2D(1 + \kappa a)} \quad (6)$$

where  $a$  is the average radius of the ions and  $\kappa$  is the reciprocal of the distance from the center of the negative ion to the center of gravity of the charge produced by the ionic atmosphere surrounding the ion.  $\kappa$  is given by

$$\kappa = \sqrt{\frac{4\pi N^2 \epsilon^2 \sum C_i Z_i^2}{1,000 DRT}} \quad (7)$$

The third step in the derivation consists in relating the electrical work to the activity coefficient of the electrolyte. The work  $W$  is exactly equal to

the free energy decrease in the system produced by the effect of the ionic charges on each other.

The free energy change in bringing a mole of a given species of ions from concentration  $C_0$  to concentration  $C_1$ , if the ions behave in an ideal fashion, is

$$\Delta F = -RT \ln \frac{C_1}{C_0} \quad (8)$$

If the ions form an ideal solution at concentration  $C_0$  but not at concentration  $C_1$ , the free energy change is

$$\Delta F = -RT \ln \frac{\gamma C_1}{C_0} \quad (9)$$

where  $\gamma$  is the activity coefficient of the ions at concentration  $C_1$ . In our particular case  $C_0$  equals  $C_1$ ; accordingly, equating  $\Delta F$  and  $W$ , we have

$$-RT \ln \gamma = \frac{NZ_1Z_2\epsilon^2\kappa}{2D(1 + \kappa a)} \quad (10)$$

When the values of the constants at 25° C. are substituted there results

$$\log \gamma = \frac{-0.509Z_1Z_2\sqrt{\mu}}{1 + 3.3 \times 10^7 a \sqrt{\mu}} \quad (11)$$

At  $\mu$  less than 0.1, equation 10 reduces to

$$\log \gamma = -0.50Z_1Z_2\sqrt{\mu} \quad (12)$$

The Debye-Hückel equation has been tested and found to yield accurate values for the activity coefficients of dilute solutions of electrolytes. It has not been found to give good results in more concentrated solutions. There are several causes for the deviations of the Debye-Hückel equation at higher concentrations. A number of approximations were made in the derivation and the dielectric constant was assumed to be independent of the electrolyte concentration.

Table 2 shows the activity coefficients of some electrolytes as determined by electromotive force measurements.

It will be noted from Table 2 that there is always a minimum in the activity coefficient-concentration relation. The Debye-Hückel equation cannot account for such a minimum; an additional term is needed, a term which increases with concentration. To explain the increase in the activity coefficients a salting-out effect is postulated. Owing to the dipolar character of water, the water molecules tend to displace ions in the vicinity of any particular ion. This, of course, is nothing but an ion hydration effect and



## IONS IN SOLUTION

TABLE 2

ACTIVITY COEFFICIENTS FROM ELECTROMOTIVE FORCE MEASUREMENTS AT 25° C.

<i>m</i>	LiCl	NaCl	KCl	KOH	HCl
0.001		0.966	0.965	0.989	
0.005		0.928	0.926	0.954	
0.01	0.901	0.903	0.899	0.920	0.91
0.05	0.819	0.821	0.815	0.822	0.836
0.1	0.779	0.778	0.764	0.789	0.801
0.5	0.725	0.678	0.644	0.750	0.762
1.0	0.757	0.658	0.597	0.760	0.823
2.0	0.919	0.670	0.569		
3.0	1.174	0.714	0.571	1.062	

results in an apparent increase of the ionic concentration. Including the salting-out effect, the Debye-Hückel equation becomes, for 25° C.,

$$-\log \gamma = \frac{0.509Z_1Z_2\sqrt{\mu}}{1 + 3.3 \times 10^7 a \sqrt{\mu}} - B\mu \quad (13)$$

where  $B$  is an empirically determined salting-out constant. Figure 1 shows a plot of the simple and extended Debye-Hückel equations.

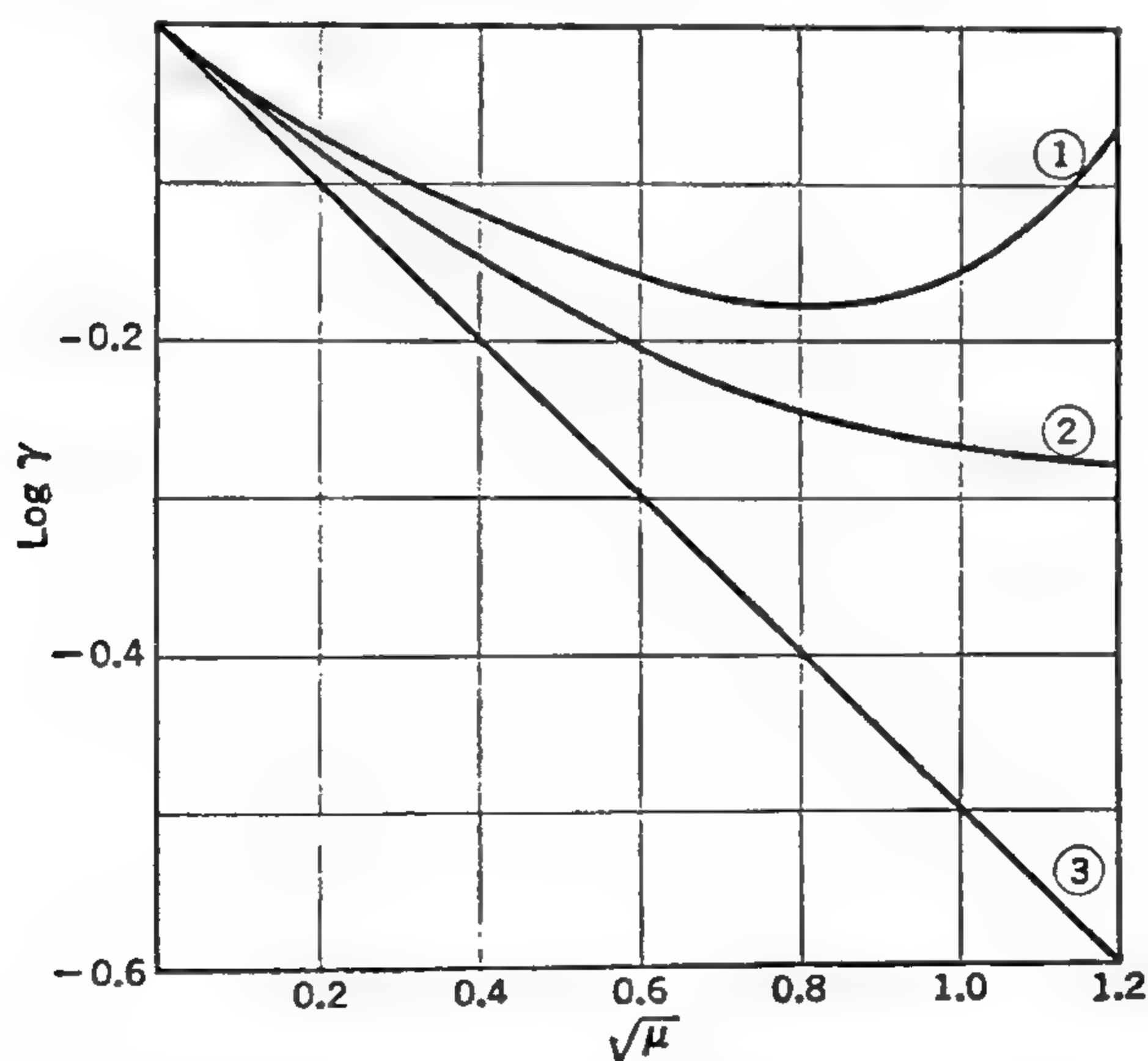


FIG. 1. Plot of the Debye-Hückel equations. (1) Equation 13. (2) Equation 10. (3) Equation 12.

Although the Debye-Hückel equations were originally derived for strong electrolytes, they are equally applicable to weak and intermediate electro-

lytes, and the ionic strength is obtained from the degree of dissociation of the weak electrolyte. In short, the Debye-Hückel equations apply to the ionized portion of the electrolyte. This topic will be discussed in greater detail in Chapter 7.

### ION HYDRATION

Ionic hydration is of considerable importance for biochemistry and is capable of explaining some of the effects of ions in living systems. In terms of the Debye-Hückel treatment such hydration refers specifically to the *B*-term in equation 13. Ionic hydration is the basis for the Hofmeister or lyotropic series of ions which we shall discuss presently.

Before we enter into the question of ion hydration, it is appropriate to indicate briefly the structure of water itself. Water is an extremely important compound for biological systems. Living tissue usually contains about 80 per cent water. The percentage may be even higher; for example, the jellyfish contains about 96 per cent water. Gortner<sup>6</sup> has rightly stressed the importance of water in biological systems.

The water molecule is V-shaped, the O—H distances being 0.96 Å and the H—O—H bond angle is 105°. Liquid water is a highly complicated structure. It was believed at one time that water was made up of definite polymers and could be considered as consisting of monohydrols, dihydrols, trihydrols, etc. X-ray studies have demonstrated that liquid water has, essentially, a broken-down ice structure and no definite polymers are present. There is, however, a marked association between the water molecules which gives rise to a quasi-ice structure. The structure of ice can be described by placing four hydrogen atoms around each oxygen in a tetrahedral distribution. Two of these hydrogen atoms belong to the same molecule as the oxygen atoms while the other two belong to adjacent oxygen atoms and point towards the oxygen atom in question. The nearest approach of water molecules in ice is 2.76 Å.

Because of the difficulty of packing tetrahedra closely, the structure of ice is very open and the density is low. The individual water molecules in ice apparently retain a considerable degree of freedom and are capable of rotation which is reflected in the relatively high dielectric constant of ice. The water molecules are bonded together by hydrogen bonds, and at low temperatures there are four such bonds per molecule corresponding to the corners of the tetrahedra. As the temperature is raised, the average number of bonds per molecule decreases, and, on fusion and further increase of temperature, more hydrogen bonds are broken until at about 40° C. the average number of hydrogen bonds per molecule is two.

<sup>6</sup> R. A. Gortner, *Outlines of Biochemistry*, John Wiley & Sons, New York, 1949.

As we have implied, increase in temperature decreases the extent of association of water. The addition of ions to water has the same effect upon the association of water as does increasing temperature; the enormous electrostatic field in the neighborhood of ions produces polarization in the water dipoles. For example, at a distance of about  $10 \text{ \AA}$  from a univalent ion, the electrostatic field has an intensity of about 14 million volts per centimeter.

Ion hydration can be expressed in terms of the energy of hydration, which is defined as that energy released when ions initially in a vacuum are placed in pure water. The energy of hydration of ions is equal to the difference between the energy required to vaporize and dissociate a mole of crystalline electrolyte into ions and the energy of solution. The energy of solution is fairly easy to arrive at by standard calorimetric procedure, but the energy of vaporization and dissociation of the crystalline salt is more difficult to obtain. It is possible to measure the concentration of alkali and halogen ions in the gas phase over a heated alkali halide crystal and to calculate the lattice energy of the crystal by standard thermodynamic methods. The lattice energy can also be calculated by means of the complicated Born-Haber cycle. The main idea of the Born-Haber cycle is to make use of the chemical heats of formation to deduce the lattice energy of the resulting crystalline compound. The cycle, which involves nine separate steps, will not be discussed further. The lattice energy of alkali halides increases as the radii of the cations or of the anions are decreased; this is to be anticipated since the electrostatic fields about the ions become more intense as the size of the ions decreases.

Bernal and Fowler<sup>2</sup> have calculated the energies of hydration of the individual ions by considering  $KF$  in which the ionic radii of the cation and anion are very nearly equal. They then make the assumption that very nearly half of the energy of hydration of  $KF$  arises from potassium ions, and thus by comparing the energies of hydration of the various salts of potassium and of fluorine they are able to assign energies of hydration to the individual ions. Shown in Table 3 are hydration values of some ions obtained in this way.

As can be seen from Table 3, as the radii of the ions for a given charge decrease the hydration energies increase. It is, however, very curious that in higher concentrations of alkali halides, the larger the radii of the anions, the greater is the depression of the vapor pressure of water at a given molar concentration.<sup>3</sup> This would seem to mean that the larger halide ions bind water more tightly than do the smaller halide ions. Although the vapor pressure data for aqueous solutions in the *International Critical Tables* are

<sup>2</sup> J. D. Bernal and R. H. Fowler, *J. Chem. Phys.* 1, 515 (1933).

<sup>3</sup> *International Critical Tables*, Vol. 3, p. 351.



rather meager, such information as we have indicates that the theoretical treatment of Bernal and Fower is incomplete. Additional experimental and theoretical work along these lines is badly needed.

TABLE 3

IONIC HYDRATION ENERGIES IN KILOGRAM CALORIES PER GRAM MOLE IONS

Ion	Hydration Energy	Ion	Hydration Energy
H <sup>+</sup>	276	Sr <sup>++</sup>	376
Li <sup>+</sup>	136	Ba <sup>++</sup>	346
Na <sup>+</sup>	114	Al <sup>+++</sup>	1149
K <sup>+</sup>	94	La <sup>+++</sup>	768
Rb <sup>+</sup>	87	Th <sup>++++</sup>	1540
Cs <sup>+</sup>	80	Ag <sup>+</sup>	162
NH <sub>4</sub> <sup>+</sup>	87	Ti <sup>+</sup>	107
OH <sub>3</sub> <sup>+</sup>	130	Mn <sup>++</sup>	479
OH <sup>-</sup>	105	Fe <sup>++</sup>	500
F <sup>-</sup>	97	Co <sup>++</sup>	504
Cl <sup>-</sup>	65	Ni <sup>++</sup>	516
Br <sup>-</sup>	57	Cu <sup>++</sup>	536
I <sup>-</sup>	47	Zn <sup>++</sup>	528
Be <sup>++</sup>	608	Cd <sup>++</sup>	462
Mg <sup>++</sup>	490	Hg <sup>++</sup>	480
Ca <sup>++</sup>	410	Fe <sup>+++</sup>	1185

Stokes and Robinson <sup>9</sup> have investigated the nature of the hydration term in the extended Debye-Hückel equation (equation 13) and have been able to express the change of the activity coefficient of the solute in terms of the effective number of water molecules associated with the ions as well as in terms of the radii of the ions. Incidentally, they note that the effective number of water molecules associated with the anions apparently increases with increasing size of the anions.

### HOFMEISTER SERIES

The biochemist has often concerned himself with what is known as the Hofmeister or lyotropic series. It is found that, when the effects of a series of cations or anions upon the salting-in or salting-out of proteins,<sup>10</sup> upon the viscosity of lyophilic colloids, upon electrokinetic potentials,<sup>11</sup> and upon a variety of other physical,<sup>12</sup> chemical, and biological properties<sup>13</sup> are compared, pronounced variations are shown. Such a series of ions is known as a Hofmeister or lyotropic series. For example, the series usually given for

<sup>9</sup> R. H. Stokes and R. A. Robinson, *J. Am. Chem. Soc.* 70, 1870 (1948).

<sup>10</sup> R. A. Gortner, W. F. Hoffman, and W. B. Sinclair, *Kolloid-Z.* 44, 97 (1928).

<sup>11</sup> D. R. Briggs, *J. Phys. Chem.* 32, 1646 (1928).

<sup>12</sup> A. Frumkin, *Kolloid-Z.* 35, 340 (1924).

<sup>13</sup> D. Glick and C. G. King, *J. Biol. Chem.* 94, 497 (1931).

## IONS IN SOLUTION

anions is citrate > tartrate >  $\text{SO}_4$  > acetate >  $\text{Cl}$  >  $\text{NO}_3$  >  $\text{Br}$  >  $\text{I}$  >  $\text{CNS}$ . The series for the cations, though usually somewhat less marked than that for the anions, is generally given as  $\text{Th}$  >  $\text{Al}$  >  $\text{H}$  >  $\text{Ba}$  >  $\text{Sr}$  >  $\text{Ca}$  >  $\text{K}$  >  $\text{Na}$  >  $\text{Li}$ . Under some conditions the series is completely reversed or the ions displaced in their position. The series is not limited to

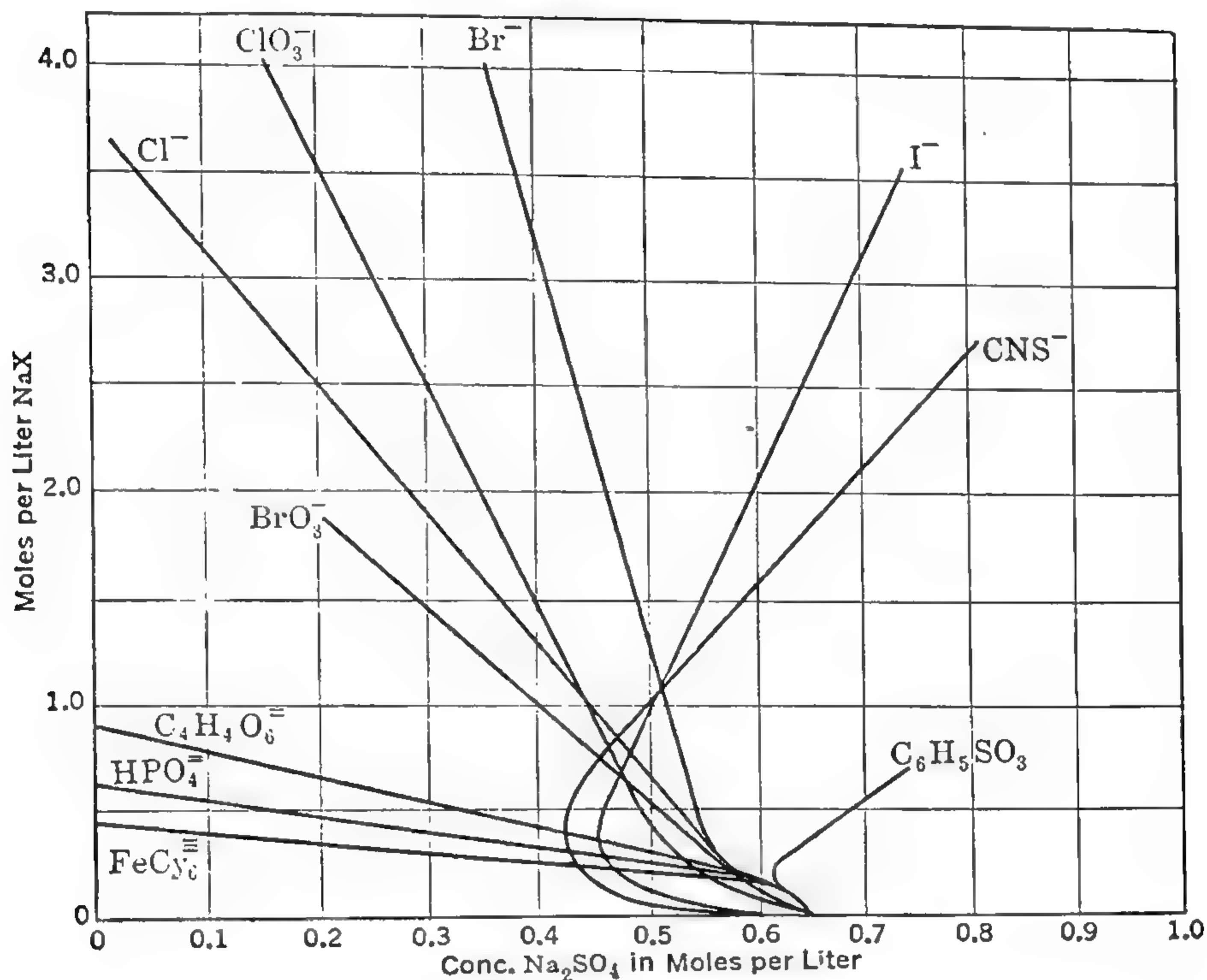


FIG. 2. Concentration of different sodium salts plotted against concentrations of  $\text{Na}_2\text{SO}_4$ , mixtures of which are necessary to bring about flocculation of agar-agar sols.

aqueous systems, as Jaeger<sup>14</sup> has found that the values of the surface tension of molten salts at  $1000^\circ\text{C}$ . fall into the series  $\text{F} > \text{SO}_4 > \text{Cl} > \text{Br} > \text{NO}_3 > \text{I}$  and  $\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs}$ .

Undoubtedly, the series has its origin in the intensity of the electrostatic field around the ions, the small ions of the same valence having a more intense field than the larger ions. As pointed out previously, the more intense field of the smaller ions leads to a greater hydration, the difference in hydration being the immediate cause of the series in aqueous systems. That this is true is shown by the success of Buchner and co-workers<sup>15</sup> in being able to

<sup>14</sup> F. M. Jaeger, *Z. anorg. allgem. Chem.* **101**, 1 (1917).

<sup>15</sup> A. Voelt, *Trans. Faraday Soc.* **32**, 1301 (1936).

<sup>16</sup> A. Voelt, *Trans. Faraday Soc.* **32**, 1301 (1936).

express the lyotropic series in quantitative terms and showing the direct relation to ionic hydration.

Buchner and co-workers studied the salting-out of gelatin and of agar-agar sols by  $\text{Na}_2\text{SO}_4$  and by mixtures of  $\text{Na}_2\text{SO}_4$  with other sodium salts. It was found that the concentration of the added sodium salt required to salt out varied linearly with the amount of  $\text{Na}_2\text{SO}_4$  required to produce the same effect. In other words, the effect of the salts was strictly additive. This is shown in Fig. 2.

A quantitative measure of the salting-out action of the ions is expressed by the angle between the lines in the figure and the abscissas. This permits the series to be expressed quantitatively by giving to every ion a number,  $N$ , which is defined by the relation

$$N = a \cot \alpha + b \tag{14}$$

where  $a$  and  $b$  are constants and  $\alpha$  is the angle formed by the salt line of the ion and the abscissas shown in the figure. The angles are different for different colloids but the number  $N$  is characteristic for every ion. To fix the scale two arbitrary numbers are used. Choosing  $\text{SO}_4^{--}$  equal to 2.00 and  $\text{Cl}^-$  equal to 10, it is found that

$$N = 4.0 \cot \alpha + 10.0 \tag{15}$$

This permits the evaluation of  $N$  for any anion. A similar series of numbers has been derived for the cations. The numbers given by Buchner are shown in Table 4.

TABLE 4  
LYOTROPIC NUMBERS OF IONS AS GIVEN BY BUCHNER ET AL.

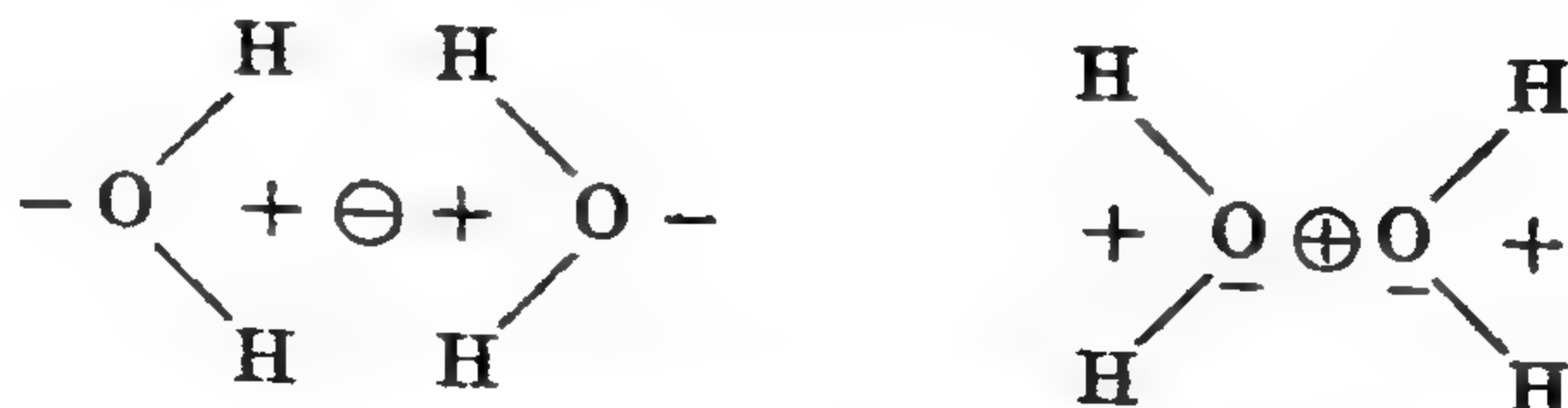
Anion	Lyotropic Number	Cation	Lyotropic Number
$\text{SO}_4^{--}$	2.0	$\text{Li}^+$	115
$\text{F}^-$	4.8	$\text{Na}^+$	100
$\text{IO}_3^-$	6.25	$\text{K}^+$	75
$\text{H}_2\text{PO}_4^-$	8.2	$\text{Rb}^+$	69.5
$\text{BrO}_3^-$	9.5	$\text{Cs}^+$	60
$\text{Cl}^-$	10.0		
$\text{NO}_2^-$	10.1		
$\text{ClO}_3^-$	10.65		
$\text{Br}^-$	11.30		
$\text{NO}_3^-$	11.60		
$\text{ClO}_4^-$	11.8		
$\text{I}^-$	12.5		
$\text{CNS}^-$	13.25		

It will be noted that, the smaller the value of  $N$ , the greater is the potency in salting out; the larger the  $N$ , the less the potency.



## IONS IN SOLUTION

It should be pointed out that cations and anions tend to orient water molecules in opposite directions



Thus, a cation carries its water with the hydrogens sticking outward. Such water can form hydrogen bonds with polar groups in a solute molecule and, accordingly, cations will tend to hydrate a colloid while the opposite effect is true of anions. A highly hydrated anion such as  $\text{SO}_4^{--}$  is a precipitating ion; the highly hydrated cation such as  $\text{Li}^+$  is a salting-in ion.

It should be remembered that the Hofmeister series is of importance only in fairly concentrated salt solutions.

## IONS IN BIOLOGY

Living material is, in general, very sensitive to its ionic content. The ionic concentrations of body fluids must be maintained within fairly narrow limits to sustain life. The body fluids of different classes of animals vary considerably in their absolute salt concentrations, the marine animals having a higher total concentration than land animals. The ratio of the various ions to one another is, however, approximately the same as that found in sea water. The most abundant cations are  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{++}$ , and  $\text{Ca}^{++}$ ; the anions are  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{SO}_4^{--}$ , and protein.  $\text{H}^+$  and  $\text{OH}^-$  ions occur in extremely small concentrations. Their roles are so important, however, that a separate chapter has been reserved for their consideration. Table 5 shows the approximate ionic composition of human blood serum.

TABLE 5

IONIC COMPOSITION OF HUMAN BLOOD SERUM

Ion	Grams per Liter	Moles per Liter	Equivalents per Liter
$\text{Na}^+$	3.25	0.1458	0.1458
$\text{K}^+$	0.19	0.0048	0.0048
$\text{Ca}^{++}$	0.10	0.0025	0.0050
$\text{Mg}^{++}$	0.027	0.0011	0.0022
$\text{HCO}_3^-$	1.64	0.0269	0.0269
$\text{Cl}^-$	3.70	0.104	0.1040
$\text{HPO}_4^{--}$	0.10	0.00105	0.0021
$\text{SO}_4^{--}$	0.19	0.00197	0.0039

Neglecting the contribution due to protein, which is small, the total ionic strength of blood is calculated to be about 0.15. This value is probably too high as some of the "ions" are undissociated. This ionic strength is just

about the upper limit of the validity of the Debye-Hückel theory, i.e., without the salting-out correction. The  $\text{Na}^+$  and  $\text{Cl}^-$  far outweigh the concentration of all the other ions put together.

The individual roles of the ions in biology are rather ambiguous and complicated. The salts have a thermodynamic role in that they help maintain the proper osmotic pressure across cell membranes. In addition to their osmotic functions, ions also have specific effects. Biological reactions occur in aqueous salt solutions and there is probably no reaction taking place in living systems that is not sensitive to some extent to the ionic environment. Possibly the most dramatic effect of ions are on enzyme systems. Thus it has been found<sup>16</sup> that  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  activate acetylcholine esterase which is the enzyme involved in the hydrolysis of acetylcholine. Acetylcholine is a substance that is liberated at the end plates of nerves following stimulation of the nerve and probably initiates muscle contraction. As contrasted with the action of  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ ,  $\text{K}^+$  inhibits acetylcholine esterase and there exists an antagonism between  $\text{Ca}^{++}$  and  $\text{K}^+$  and also between  $\text{Mg}^{++}$  and  $\text{K}^+$ . The presence of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  increases the activity of the enzyme even in the presence of  $\text{K}^+$ .

Smith<sup>17</sup> has emphasized the importance of metal ions such as cobalt, magnesium, and manganese on the activation of peptidases and has published a very stimulating theory of their role. He believes that those peptidases that are activated by metal ions form a chelate ring with the metal, peptide, and enzyme. Such ring formation leads to a weakening of the peptide bond with subsequent hydrolysis of this bond. It is well known that many of the oxidation-reduction enzymes also require metal ions to become active.

An extensive review of the role of metal ions in enzyme systems has been published by Lehninger.<sup>18</sup>

The absolute salt concentration in a living system is not so important as the relative concentration of ions. Thus sodium in high concentrations is toxic, but the toxicity largely disappears if the increased concentration is accompanied by a corresponding increase in the concentration of the other ions. Ions frequently exhibit an antagonistic action. A striking antagonism is shown between  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ . Meltzer and Auer<sup>19</sup> showed that, if magnesium salt is injected into the blood stream of vertebrates, a profound anesthesia results in which the animals become motionless and insensitive to pain. If into such an anesthetized animal a trace of calcium

<sup>16</sup> B. Mendel, D. Mundell, and F. Strelitz, *Nature* 144, 479 (1939).

<sup>17</sup> E. L. Smith, *Proc. Natl. Acad. Sci. U. S.* 35, 80 (1949).

<sup>18</sup> A. L. Lehninger, *Physiol. Revs.* 30, 393 (1950).

<sup>19</sup> S. J. Meltzer and J. Auer, *Am. J. Physiol.* 14, 366 (1905); 16, 233 (1906); 21, 400 (1908).



salt is injected, there is a prompt awakening and return to sensitivity. Seifriz<sup>21</sup> has commented on the action of ions on living systems correlating their action with some of the chemical properties of ions.

#### PENETRATION OF CELLULAR MEMBRANES BY IONS

The important problem of the penetration of living cells by ions has been the subject of exhaustive study,<sup>21-23</sup> and the amount of detailed information available is overwhelming. No unified picture has, however, emerged from this vast accumulation of knowledge. In this short account we shall attempt to outline the problem very briefly, recognizing that any exposition in the short space at our disposal is necessarily inadequate.

It is essential to determine if a given accumulation or secretion of ions is a spontaneous process or one that requires work to be done. The answer to this question must be found in the free energy changes involved. If we consider all ions capable of being transferred across the cell membrane, the free energy change per mole of ions transferred will be

$$\Delta F = \frac{RT}{Z_A} \ln \frac{A_2}{A_1} + \frac{RT}{Z_B} \ln \frac{B_2}{B_1} + \cdots + \frac{RT}{Z_N} \ln \frac{N_2}{N_1} \quad (16)$$

where  $A_2$ ,  $B_2$ ,  $N_2$ , etc., refer to the activity of the various ions inside the cell,  $A_1$ ,  $B_1$ ,  $N_1$ , etc., refer to the activity of the various ionic species in the solution bathing the cell, and  $Z_A$ ,  $Z_B$ ,  $Z_N$ , etc., refer to the respective valences of the ions. Exact calculations require a knowledge of the ionic activities, but the accuracy of measurement seldom justifies this refinement and concentrations can usually be employed. If  $\Delta F$  is zero, the system is at equilibrium (the situation of a Donnan equilibrium). If it is negative, the accumulation of ions is spontaneous and requires no work on the part of the cell. If, on the other hand, it is positive, it means that the cell has had to do work to bring about the transfer, the minimum value of which is given by  $\Delta F$  in calories. In general, such a process is not very efficient and several times the work calculated is actually required. One of the pressing unsolved problems of physiology is how the cell does any work—work of any kind. The problem of heat production has been extensively investigated and is in large measure understood; but how the body transforms chemical energy into mechanical or into osmotic work is almost a complete blank.

<sup>21</sup> Wm. Seifriz, *Science* 110, 193 (1949).

<sup>22</sup> R. Hober, *Physical Chemistry of Cells and Tissues*, The Blakiston Co., Philadelphia, 1945.

<sup>23</sup> H. Davson and J. F. Danielli, *The Permeability of Natural Membranes*, The Macmillan Co., New York, 1943.

<sup>24</sup> H. H. Ussing, *Physiol. Revs.* 29, 127 (1949).



Rosenberg<sup>24</sup> defines active ion transport as transfer that takes place from a lower to a higher electrochemical potential. The electrochemical potential difference for an ionic species between two dilute solutions at the same pressure is equal to  $RT \ln A_1/A_2 + ZF\psi_1 - \psi_2$ , where  $\psi_1 - \psi_2$  is the electrical potential difference between them and  $Z$  is the valence of the ion. This definition is equivalent to the one given in equation 16. Both these definitions are operationally difficult to apply in a living system. However, there can hardly be any doubt that in many living systems active ion transport does indeed occur. For example, it can be estimated<sup>25</sup> that about 1500 calories are required for the secretion of one liter of gastric juice by the parietal cells of the stomach mucosa. Borsook and Winegarden<sup>26</sup> have calculated that the kidney does about 700 calories of work in the secretion of a liter of urine. The concentration of potassium ions in the cell sap of the marine alga *Valonia macrophysa* is about 500 meq. per liter against 12 meq. per liter in the surrounding sea water.<sup>27</sup> There can be no question that this concentration of potassium inside the *Valonia* represents an active transport of potassium ions. Many other examples could be given.

The active transport of an ion implies a specific mechanism for this transport, and the energy for this process is furnished by cellular metabolism. Osterhout and Stanley<sup>28</sup> undertook to devise an artificial homogeneous membrane which would simulate some of the properties of living membranes. They found that a mixture of 70 per cent guaiacol and 30 per cent *p*-cresol fulfilled their expectations to a surprising degree. It has been reported, for example, that the giant plant cell *Valonia* accumulates potassium and sodium from sea water, the potassium being in greater concentration than the sodium. The homogeneous membrane of Osterhout also accumulates potassium and sodium. The arrangement of the model membrane is shown in Fig. 3.

The KOH in *A* reacts with the guaiacol to give the potassium salt. This potassium salt then diffuses until it comes into contact with *C* and potassium bicarbonate is produced and is released into compartment *C*. This

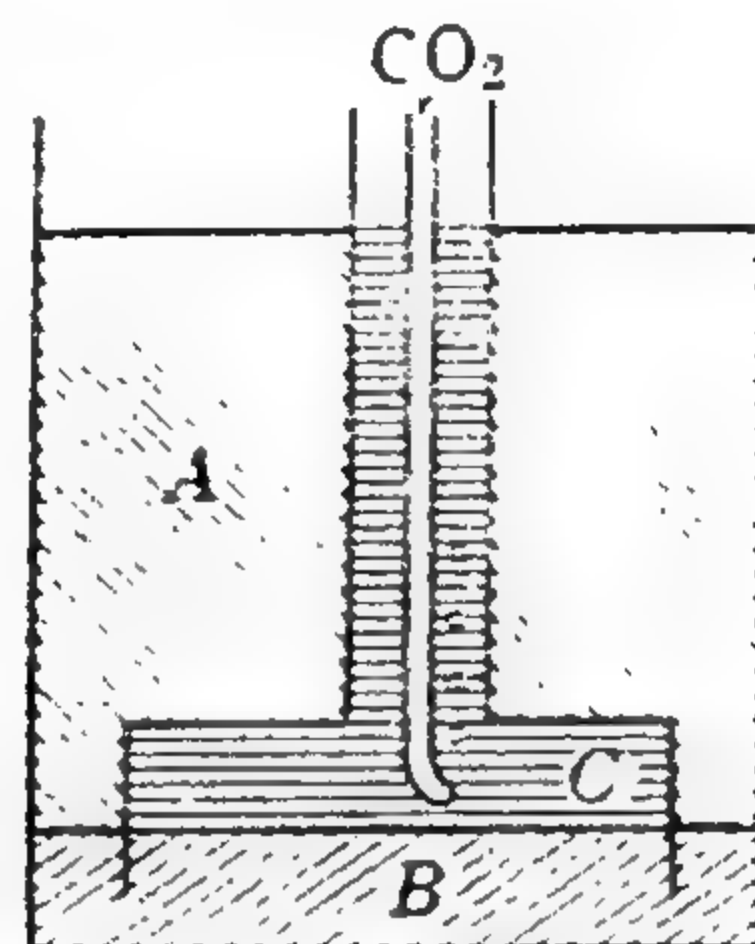


FIG. 3. Osterhout-Stanley model for a living membrane. *A* is a 0.04 *M* KOH solution. *B* is a non-aqueous layer of *p*-cresol and guaiacol. *C* is water into which  $\text{CO}_2$  gas is bubbled.

<sup>24</sup> T. Rosenberg, *Acta Chem. Scand.* 2, 14 (1948).

<sup>25</sup> H. B. Bull and J. S. Gray, *Gastroenterology* 4, 175 (1945).

<sup>26</sup> H. Borsook and H. M. Winegarden, *Proc. Natl. Acad. Sci. U. S.* 17, 313 (1931).

<sup>27</sup> L. R. Blinks, *Cold Spring Harbor Symposia Quant. Biol.* 8, 204 (1940).

<sup>28</sup> W. J. V. Osterhout and W. M. Stanley, *J. Gen. Physiol.* 15, 667 (1932).

leads to an accumulation of potassium in compartment C, the accumulation being brought about through the action of  $\text{CO}_2$ . The  $\text{CO}_2$  is supposed to represent the end products of metabolism. Figure 4 shows a graph of some of their results using 0.05 *N* NaOH and 0.05 *M* KOH in compartment A.

Although the Osterhout model does not reproduce all the ramifications of electrolyte penetration into living cells, it is useful in demonstrating one

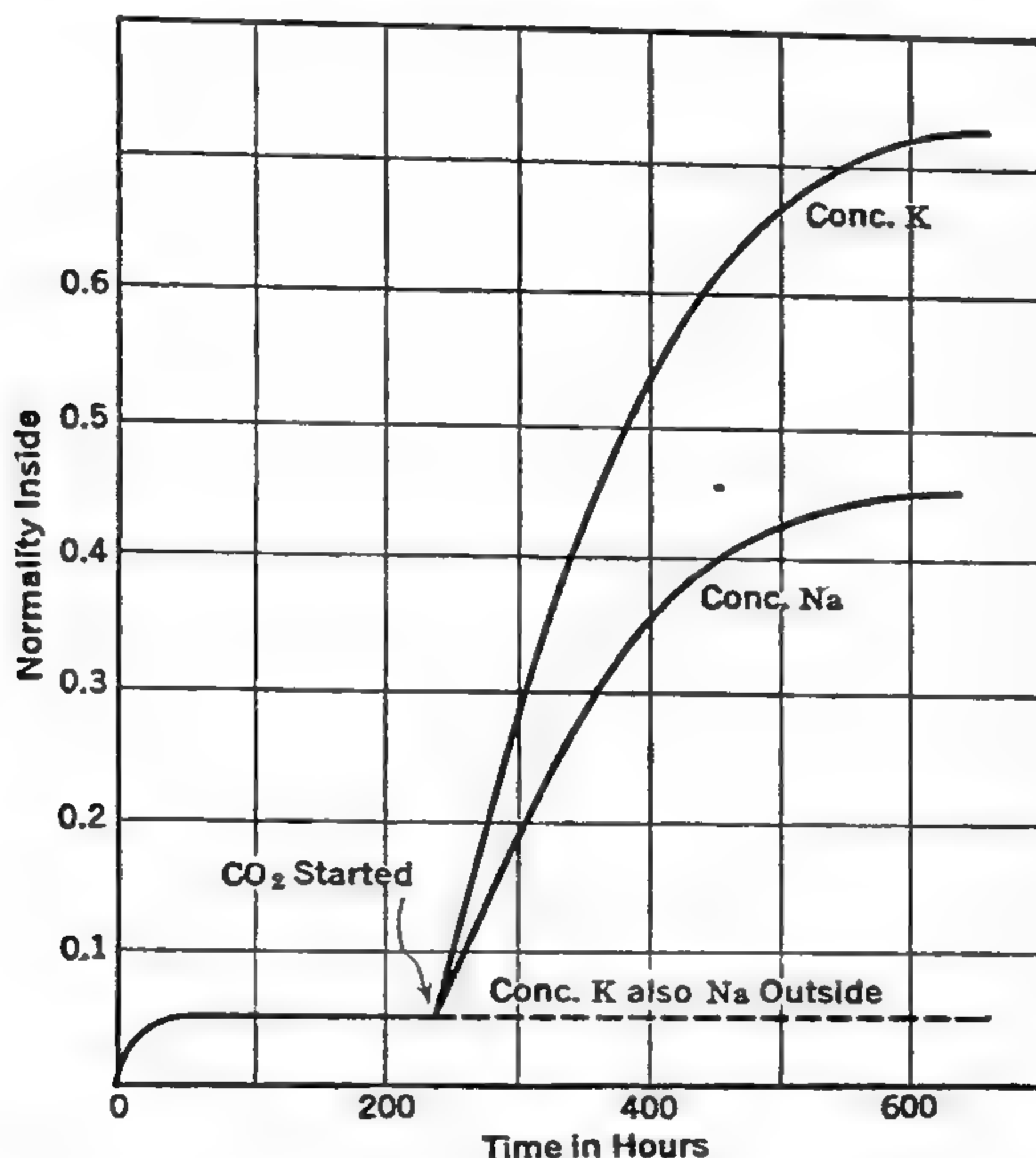


FIG. 4. Graph showing accumulation of sodium and potassium in compartment C (see FIG. 3) due to the presence of  $\text{CO}_2$ . (Osterhout and Stanley.)

of the outstanding features of such penetration, namely, the dependence of electrolyte accumulation on an active mechanism which is represented in the model by a continuous supply of  $\text{CO}_2$ .

Living cells are capable of producing both hydrogen ions and anions. That is, by the process of metabolism the cell can oxidize some non-ionogenic organic compound to an organic acid. It can then, in principle, exchange the proton from the carboxyl group of the organic acid for another cation. Conway and Brady<sup>29</sup> have found that yeast cells exchange hydrogen ions for potassium ions and in this instance the hydrogen ions originate from succinic acid. It is possible to offer an explanation of the secretion of hydrochloric acid by the stomach based on an ion exchange mechanism.<sup>25</sup>

<sup>29</sup> E. J. Conway and T. Brady, *Nature* 159, 137 (1946).

There exists another mechanism for active ion transport in addition to ion exchange. For example, Lundegardh<sup>30</sup> has suggested that anion transport may be brought about by a valency change in the iron in the hemin group of a respiratory enzyme when the iron is oxidized from the ferrous to the ferric state; the trivalent iron attracts one more anion than does the bivalent iron. It is assumed that a series of iron enzymes is arranged across a cell membrane and that there exists a difference of oxidation-reduction potential between the inside and the outside of the cell. Such an arrangement could result in transport of anions from the medium of higher oxidation potential to the medium with the lower oxidation potential. However, such a system would likewise, by necessity, transport hydrogen ions. Every electron arriving at the oxidation side requires the neutralization of one positive charge and the disappearance of one hydrogen ion, and the formation of the electron at the reduction side means the formation of hydrogen ions. This is essentially the process that Davies<sup>31</sup> postulates for the production of hydrochloric acid by the stomach mucosa.

The suggestion has been made that an electrical potential difference between the inside and the outside of the cell can lead to active ion transport. Now, the difference in potential across a cell membrane arises directly from the difference in ion concentration across the membrane. The effect of the electrical potential would always be in the direction of decreasing the difference in ion concentration and tend to force the system towards a state of ionic equilibrium and away from active ion transport.

We have outlined a few of the possible mechanisms for active ion transport; these have a general and not a specific character. One of the outstanding features of active ion transport, however, is exactly this specificity. For example, living cells tend to accumulate potassium ions and to secrete sodium ions. The nature of this specificity is quite obscure.

## PROBLEMS AND QUESTIONS

1. What rare gas structures correspond to the following ions:  $\text{Li}^+$ ,  $\text{F}^-$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$ ?
2. Calculate the ionic strength of a 0.1 molar solution of  $\text{Na}_2\text{HPO}_4$ . Ans.: 0.30.
3. Calculate the value of  $\kappa$  at 25° C. for a 0.01 molar  $\text{CaCl}_2$  solution. Ans.:  $5.68 \times 10^6$  reciprocal centimeters.
4. Calculate the activity coefficient of a 0.01 molar  $\text{CaCl}_2$  solution. Ans.: 0.67.

<sup>30</sup> H. Lundegardh, *Arch. Botanik* 32A, No. 12 (1945).

<sup>31</sup> R. E. Davies, *Biochem. J.* 42, 609 (1948).



## ELECTRICAL CONDUCTANCE

It is evident that, if electrolytes exist partly or completely as ions in solution, and if a difference of potential exists across two electrodes placed in such a solution, a current will flow between the electrodes. This flow of current is due to the migration of ions through the solution. The cations, being positively charged, travel towards the negative electrode (cathode), and the anions, having a negative charge, move towards the positive electrode (anode). The amount of current carried by an ion will depend on its valence and on its speed of migration. One gram equivalent of an ion will transport 96,404 coulombs of electricity.

## EXPERIMENTAL DETERMINATION OF CONDUCTANCE

The specific resistance of any substance, whether in solution or not, may be defined as the resistance in ohms of a column of substance 1 centimeter long and 1 square centimeter in cross section. Specific conductance (or conductivity) is the reciprocal of the specific resistance and is expressed in reciprocal ohms or mhos. Conductance is defined as the ratio of the current flowing through a conductor to the difference in potential between its ends. Conductivity, as we have seen, can be defined as the quantity of electricity transferred across unit area per unit potential gradient per unit time.

The determination of the conductance of a solution is one of the simplest and most accurate of physical measurements. A conductance cell is employed which consists of a glass vessel of the proper shape and dimensions with two platinum disks held in fixed positions in the cell. The platinum electrodes are covered with platinum black. The cell is calibrated by measuring the resistance offered to a solution of known conductance (usually 0.1 or 0.01 *N* KCl). The unknown is then placed in the cell, its resistance is measured, and the specific conductance of the solution is calculated. For example, suppose that the resistance of the cell filled with 0.01 *N* KCl is 3.70 ohms at 25° C. The specific conductance of 0.01 *M* KCl at 25° C. is

0.001407. Accordingly, the cell constant is  $307.9 \times 0.0014$  or 0.428. In order to find the specific conductance of the unknown, we divide the cell constant by the resistance offered by the unknown solution or

$$\text{Specific conductance } (\kappa) = \frac{\text{Cell constant}}{\text{Resistance}} \quad (1)$$

The proper shapes and sizes of the conductance cell are given by Washburn<sup>1</sup> and by Jones and Josephs;<sup>2</sup> the best values for the calibrating solutions by Jones and Bradshaw.<sup>3</sup>

The electrical apparatus for measuring conductance makes use of a Wheatstone bridge, shown in Fig. 1.

The resistance of the cell is measured by balancing its resistance against that of the known  $R_1$  by moving the slide along  $S$  until no sound is heard in the receiver  $T$ . The resistance of the cell is then calculated by the following proportion

$$\frac{R_2}{R_1} = \frac{ab}{bc} \quad (2)$$

where  $R_2$  is the resistance of the conductance cell,  $R_1$  the known resistance, and  $ab$  and  $bc$  refer to the position of the slide wire at balance. Various modifications of this simple arrangement have been employed. The electrodes of the conductance cell form a condenser with a certain capacity, and, in order to compensate for this capacity, a condenser of approximately the same capacity should be included in parallel with the known resistance. The proper design for a Wheatstone bridge is described by Jones and Josephs.<sup>3</sup> A very satisfactory method for solutions whose conductance is not too high is to replace receiver  $T$  by an alternating-current galvanometer, and the buzzer  $B$  by a source of 60-cycle alternating current of low voltage. The slide is fixed at the mid-point of the slide wire, and balance is obtained by varying  $R_1$  until no deflection in the alternating-current galvanometer is observed. The resistance of the cell is then exactly equal to the known resistance.

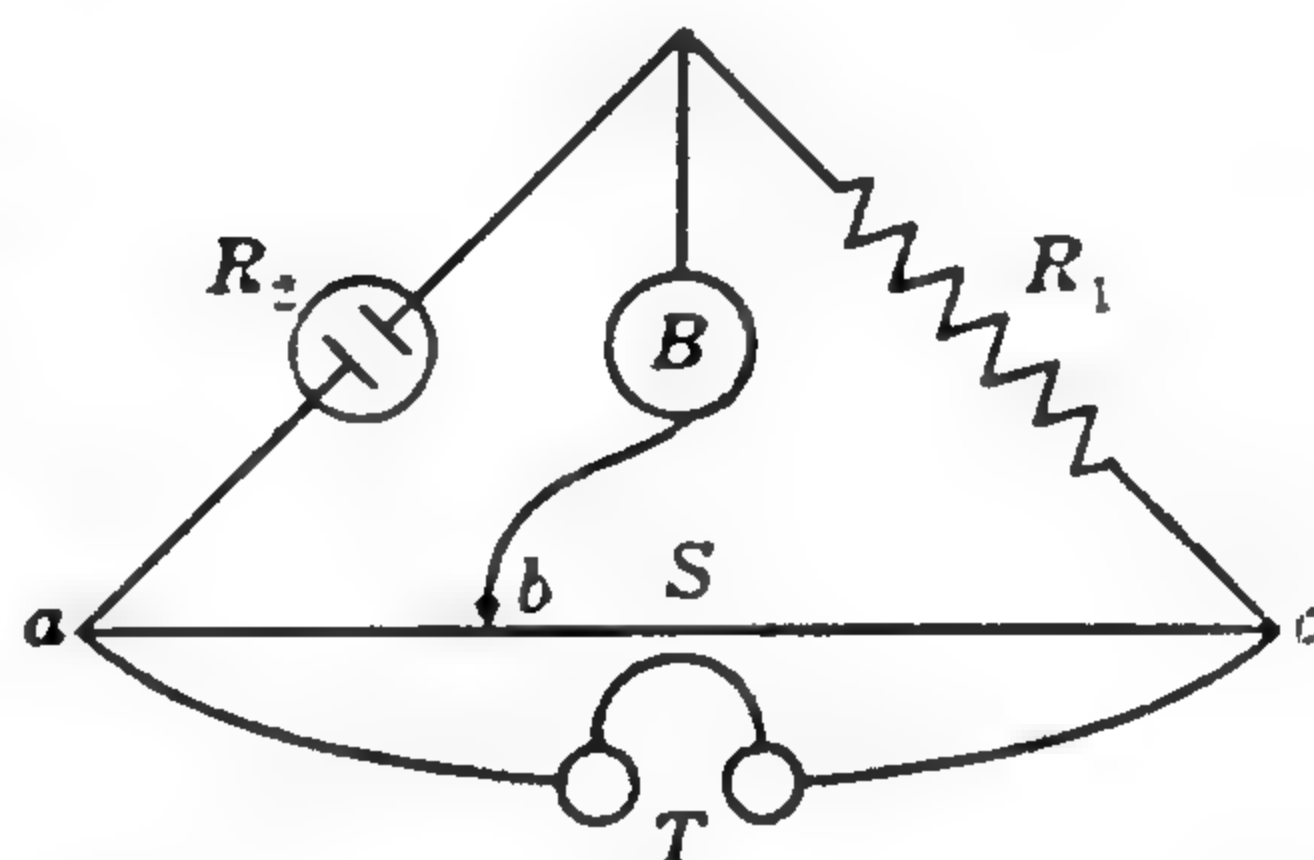


FIG. 1. Wheatstone bridge.  $R_1$  is a known resistance;  $S$ , a graduated resistance slide wire;  $R_2$ , resistance of the conductance cell;  $B$ , a buzzer giving an alternating current (usually 1000 cycles per second);  $T$ , a telephone receiver.

<sup>1</sup> E. W. Washburn, *J. Am. Chem. Soc.* 38, 2431 (1916).

<sup>2</sup> G. Jones and R. C. Josephs, *J. Am. Chem. Soc.* 50, 1049 (1928).

<sup>3</sup> G. Jones and B. C. Bradshaw, *J. Am. Chem. Soc.* 55, 1780 (1933).

## METHODS OF EXPRESSION

Other ways of expressing conductance are by equivalent and by molar conductance. The equivalent conductance is the conductance in reciprocal ohms of a solution containing 1 gram equivalent of the electrolyte enclosed between two electrodes 1 centimeter apart. Similarly, molar conductance is the conductance in mhos of a solution containing 1 gram molecular weight under the same conditions. The relation between the specific conductance, equivalent conductance, and molar conductance is as follows: If  $\kappa$  denotes the specific conductance of a solution,  $\Lambda$  the equivalent conductance,  $\mu$  the molar conductance,  $V_e$  the volume in cubic centimeters of solution which contains 1 equivalent of solute, and  $V_m$  the volume which contains 1 gram molecular weight of the solute, we have

$$\mu = \Lambda V_m \quad (3)$$

and

$$\Lambda = \kappa V_e \quad (4)$$

If  $C$  denotes the concentration of a solution of an electrolyte in gram equivalents per liter, we have

$$V_e = \frac{1,000}{C} \quad (5)$$

Substituting equation 5 in equation 4, we obtain

$$\Lambda = \frac{1,000\kappa}{C} \quad (6)$$

A similar relation can be developed for the molar conductance.

The specific conductance (or conductivity) of an electrolyte decreases and approaches that of the solvent as the concentration is decreased, whereas the equivalent conductance and the molar conductance increase with decreasing concentration and approach a limit which is characteristic of the solute ions. The variation of the conductance with electrolyte concentration is due to two influences: (1) increased ionization with decreased concentration, and (2) decreased interaction of the ions upon dilution. The first effect was one of the strongest supports for Arrhenius' theory of ionization. The dependence of the equivalent conductance on electrolyte concentration can be formulated quantitatively and used to evaluate the ionization constants of weak electrolytes. The second effect has been considered by Debye and Hückel<sup>4</sup> and in more detail by Onsager.<sup>5</sup> The

<sup>4</sup> Debye and Hückel, *Physik. Z.* 24, 185, 305 (1923).

<sup>5</sup> L. Onsager, *Physik. Z.* 27, 388 (1926).



theoretical treatment, however, is too involved for us to consider. For a fairly simple exposition of this effect, see a paper by MacInnes.<sup>4</sup>

The ionization "constant" of a weak electrolyte can be obtained from the equation

$$K = \frac{\Lambda^2 C}{\Lambda_0(\Lambda_0 - \Lambda)} \quad (7)$$

where  $C$  is the concentration in gram equivalents per liter,  $\Lambda$  is the equivalent conductance at the given concentration,  $\Lambda_0$  is the equivalent conductance at infinite dilution (the limiting conductance as the electrolyte is made more and more dilute), and  $K$  is the ionization constant, the value of which, however, varies somewhat with concentration. As the dilution is increased, the constant approaches the true constant more closely. (See Chapter 7 for a discussion of the ionization constants of weak acids.)

The equivalent conductance of a dilute solution of a strong electrolyte (completely ionized) varies inversely as the square root of the concentration.

The specific conductance of an aqueous solution increases approximately 2 per cent for each degree rise in temperature. This is a rather complicated effect but involves principally the decrease in the viscosity of the water with increasing temperature.

## INDEPENDENT MIGRATION OF IONS

The conductance of an electrolyte solution is due to the migration of both the cations and the anions. At infinite dilution the migration of cation and anions is independent of each other (the interaction of the ions with each other has been reduced to zero). Under these conditions

$$\Lambda_0 = l_+ + l_- \quad (8)$$

where  $l_+$  and  $l_-$  are the ion conductances, at infinite dilution of the cation and anion, respectively.

The quantity of positive electricity carried by the cations in one direction is proportional to their speed  $u$ , and the quantity of negative electricity moving in the opposite direction is proportional to  $v$ , the speed of the anions: the total current carried is then proportional to  $u + v$ . The fraction of the total current carried by the cation is, therefore,  $u/(u + v)$ . This fraction is called the transport or transference number of the cation. Similarly the transport number of the anion is  $v/(u + v)$  and the sum of the transport numbers of the cations and anions is unity. As a result of the difference in the velocities of the two ions, different concentration changes

<sup>4</sup> D. A. MacInnes, *Science* 86, 23 (1937).

## ELECTRICAL CONDUCTANCE

occur in the neighborhood of the cathode and anode, respectively, and these changes may be utilized to evaluate transport numbers.

Evidently at infinite dilution the transport number of the cations is  $l_+/(l_+ + l_-)$ , and if we combine this result with equation 8 we have the possibility of calculating  $l_+$  and  $l_-$  from a knowledge of the transport numbers extrapolated to infinite dilution and from the equivalent conductance at infinite dilution.

Now,  $l_+$  and  $l_-$  represent the coulombs of electricity conducted per sec. under a potential gradient of 1 volt per centimeter. If we divide the  $l_+$  and  $l_-$  values by  $F$  (96,500 coulombs) the result will be the absolute mobilities of the cation and anion, respectively, at infinite dilution. Table 1 shows the ionic mobilities at 25° C. at infinite dilution calculated in this manner.

TABLE 1  
VELOCITIES OF IONS AT 25° C.

Ion	Velocity in cm./sec./volt./cm.	Ion	Velocity in cm./sec./volt./cm.
H <sup>+</sup>	36.2 × 10 <sup>-4</sup>	Cl <sup>-</sup>	7.92 × 10 <sup>-4</sup>
Cs <sup>+</sup>	8.00 × 10 <sup>-4</sup>	Br <sup>-</sup>	8.13 × 10 <sup>-4</sup>
K <sup>+</sup>	7.61 × 10 <sup>-4</sup>	I <sup>-</sup>	7.97 × 10 <sup>-4</sup>
NH <sub>4</sub> <sup>+</sup>	7.60 × 10 <sup>-4</sup>	NO <sub>3</sub> <sup>-</sup>	7.40 × 10 <sup>-4</sup>
Na <sup>+</sup>	5.20 × 10 <sup>-4</sup>	OH <sup>-</sup>	20.5 × 10 <sup>-4</sup>
Li <sup>+</sup>	4.01 × 10 <sup>-4</sup>	CH <sub>3</sub> COO <sup>-</sup>	4.25 × 10 <sup>-4</sup>
Ca <sup>++</sup>	6.16 × 10 <sup>-4</sup>	SO <sub>4</sub> <sup>—</sup>	8.28 × 10 <sup>-4</sup>

As can be seen from Table 1, the cationic velocities can be arranged in a lyotropic series; for the alkali metal ions, the smaller the ion the smaller is its mobility. This somewhat anomalous behavior is explained as the basis of hydration; the smaller cations are hydrated to a greater extent owing to their more intense electrostatic fields. This hydration increases their effective sizes and accordingly decreases their mobility. No such simple regularity is observed in the case of the anions.

Sometimes confusion arises over the fact that equivalent quantities of ions are liberated at the two electrodes in spite of the fact that, in general, the fraction of the total current carried by the cation is not equal to that transported by the anion. Consider the extreme case in which the mobility of the anion would be zero and all the current would be carried by the cation. Under these conditions the cations would move out of the anode towards the cathode and there would be an accumulation of cations at the cathode. There would be an equivalent excess of anions at the anode because cations had migrated out of the anode. Thus there would be unpaired cations at the cathode and an equivalent quantity of unpaired

anions at the anode, and these unpaired ions would be discharged at the respective electrodes.

## USES OF CONDUCTANCE MEASUREMENTS

There are a variety of uses for conductance measurements in the biochemical laboratory. The end point of acid-base titrations can be accurately determined. The conductance shows a sharp break at neutralization. Both hydrogen and hydroxyl ions have a high mobility; accordingly, at neutralization the conductance changes sharply.

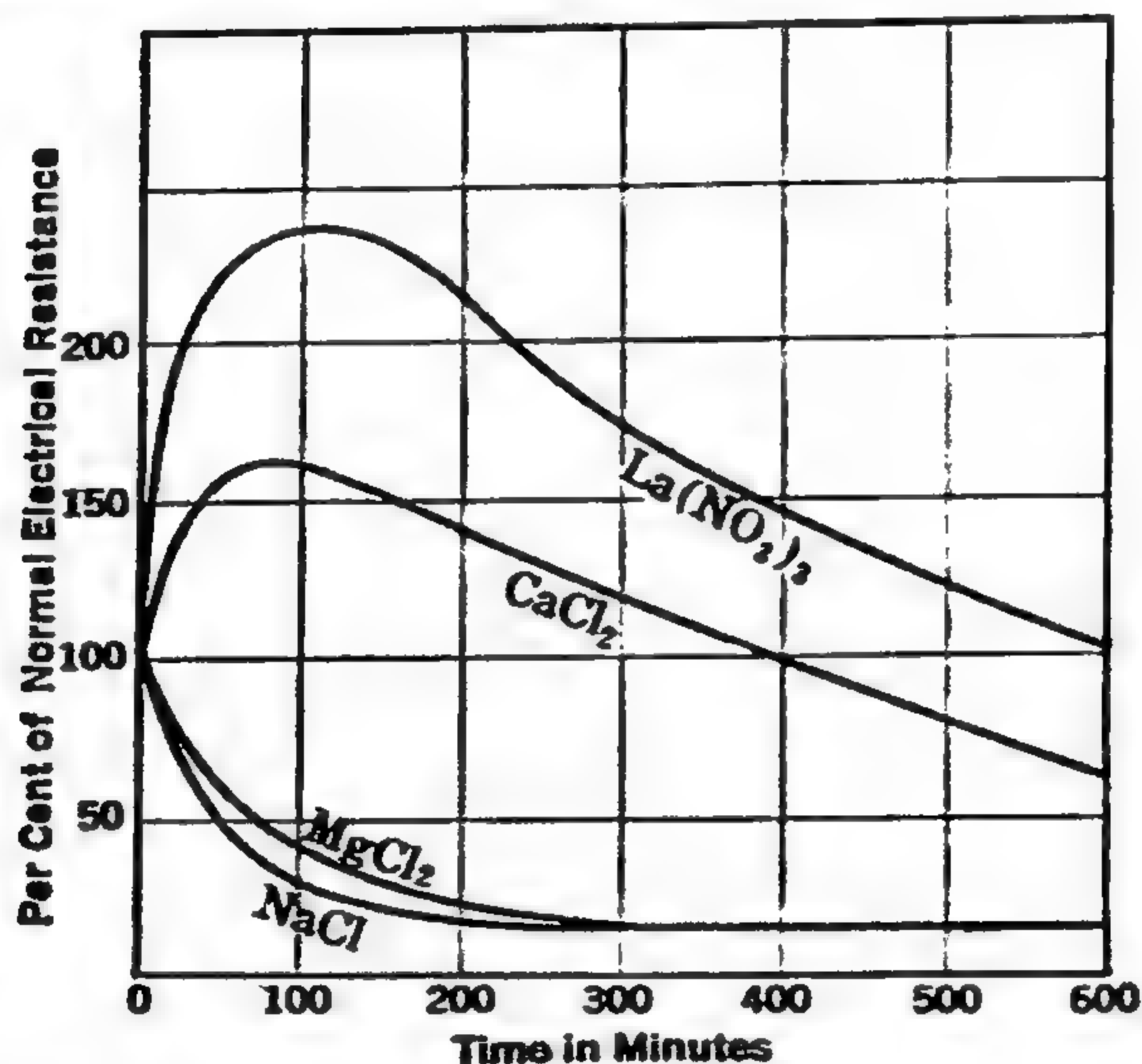


FIG. 2. Conductance of *Laminaria agardhii* in the presence of various ions. (Osterhout.)

Conductance measurements furnish a very convenient way of testing for the absence or presence of electrolytes in protein preparations.

Conductance measurements have been extensively used to study permeability changes in living cells. Obviously, an increase in the conductance of a cell means that ions are passing through the cell with greater ease. Osterhout was a pioneer in the study of living-cell permeability by means of conductance. Figure 2 shows some of his results on the sea alga *Laminaria agardhii*.<sup>7</sup>

The electrolyte concentrations used in Osterhout's experiments were such as to yield the same conductivity as sea water. In the early stages of the experiments the process is reversible; but, if there is too great a change in conductivity, either increasing or decreasing, the process is irreversible

<sup>7</sup> W. J. V. Osterhout, *Injury, Recovery and Death in Relation to Conductivity and Permeability*, J. B. Lippincott Co., Philadelphia, 1922.



and death results. The resistance of normal *Laminaria* is about 10 times that of sea water, whereas the dead tissue has a resistance about equal to that of sea water. In general,  $\text{Ca}^{++}$  decreases the permeability and  $\text{Mg}^{++}$ ,  $\text{Na}^+$ , and  $\text{K}^+$  increase it.

One of the difficulties of conductance measurements on cell suspensions is that current passes not only through the cells but also around them. This source of confusion is resolved by applying Maxwell's equation which describes the resistance of suspensions of conducting spheres in a conducting suspension. This equation is

$$\frac{\frac{r_1}{r} - 1}{\frac{r_1}{r} + 2} = \phi \left( \frac{\frac{r_1}{r_2} - 1}{\frac{r_1}{r_2} + 2} \right) \quad (9)$$

where  $r$  is the specific resistance of the suspension,  $r_1$  the specific resistance of the suspending liquid,  $r_2$  the specific resistance of the suspended particles, and  $\phi$  the ratio of the volume of the suspended particles to the total volume of the suspension.

The conductance of suspensions of ellipsoids has been discussed by Fricke<sup>8</sup> and more recently by Velick and Gorin.<sup>9</sup> These last workers derived an equation for non-conducting ellipsoids which takes the form

$$\phi = \frac{\frac{r}{r_1} - 1}{\frac{r}{r_1} - 1 + f} \quad (10)$$

where  $f$  is a form factor. For spheres it is 1.5, and for all other structures it is greater than 1.5.

To use this equation, the ratio of  $r$  to  $r_1$  of a relatively concentrated suspension is measured. The suspension is then diluted by the addition of known amounts of the medium, and the ratio  $r/r_1$  is determined for each mixture. The form factor  $f$  and  $\phi$  are then obtained in the following way: Let  $\phi'$  and  $r'/r_1'$  be the volume fraction and conductance ratios, respectively, for the most dilute member of the dilution series, and  $\phi$  and  $r/r_1$  the same for any other member of the series. Let  $V_1$  be the total volume to which 1 cc. of the original suspension was diluted for the most dilute member of the series, and  $V$  the total volume containing 1 cc. of the original

<sup>8</sup> H. Fricke, *Cold Spring Harbor Symposia Quant. Biol.* **1**, 117 (1933).

<sup>9</sup> S. Velick and M. Gorin, *J. Gen. Physiol.* **23**, 753 (1940).

suspension for any other member of the series. With this arrangement, it follows from equation 10 that

$$\frac{V}{V_1} = \phi' \left( 1 + \frac{f}{(r/r_1) - 1} \right) \quad (11)$$

When  $\frac{V}{V_1}$  is plotted against  $\frac{1}{(r/r_1) - 1}$ , a straight line should be obtained with the intercept  $\phi'$  and a slope of  $\phi'f$  from which  $\phi'$  and  $f$  are found. Velick and Gorin verified their equation with duck erythrocytes.

Much work has been done on the conductance of biological systems (individual cells, cell suspensions, and tissue cells), using varying high-frequency currents (500 to 10 million cycles).

In general, as the frequency is increased, the resistance of biological systems decreases. In order to understand the apparent drop in resistance of biological systems with increasing frequency, it is necessary to examine for a moment in a qualitative fashion the behavior of alternating currents through various circuits. If a condenser is connected in series with a direct-current source, there is a momentary flow of current due to the charging of the condenser. After the condenser is charged, however, the flow of current in a perfect condenser stops completely. On the other hand, if a condenser is placed in series in an alternating circuit, there is a flow in

and out of the condenser and, as the frequency is increased, the apparent flow of current increases to reach a limiting value. Evidently, there will be a time lag between the maximum current and the maximum potential, since the condenser is charged before the potential reaches a maximum. For a perfect condenser (no leakage), the maximum current is a quarter cycle ahead of the maximum potential. The term reactance is applied to the ratio of the maximum potential to the maximum current.

For a pure resistance, the maximum current and maximum potential naturally coincide. In most actual circuits there is a mixture of capacity and resistance. Accordingly, the phase angle between the potential and the current may be anywhere from  $0^\circ$  to  $90^\circ$ . The ratio of the maximum values of the potential to the current in such a mixed circuit is called the impedance. In biological systems we deal, in general, with circuits involving both resistance and capacity. Such a situation can be represented by an equivalent circuit (Fig. 3).

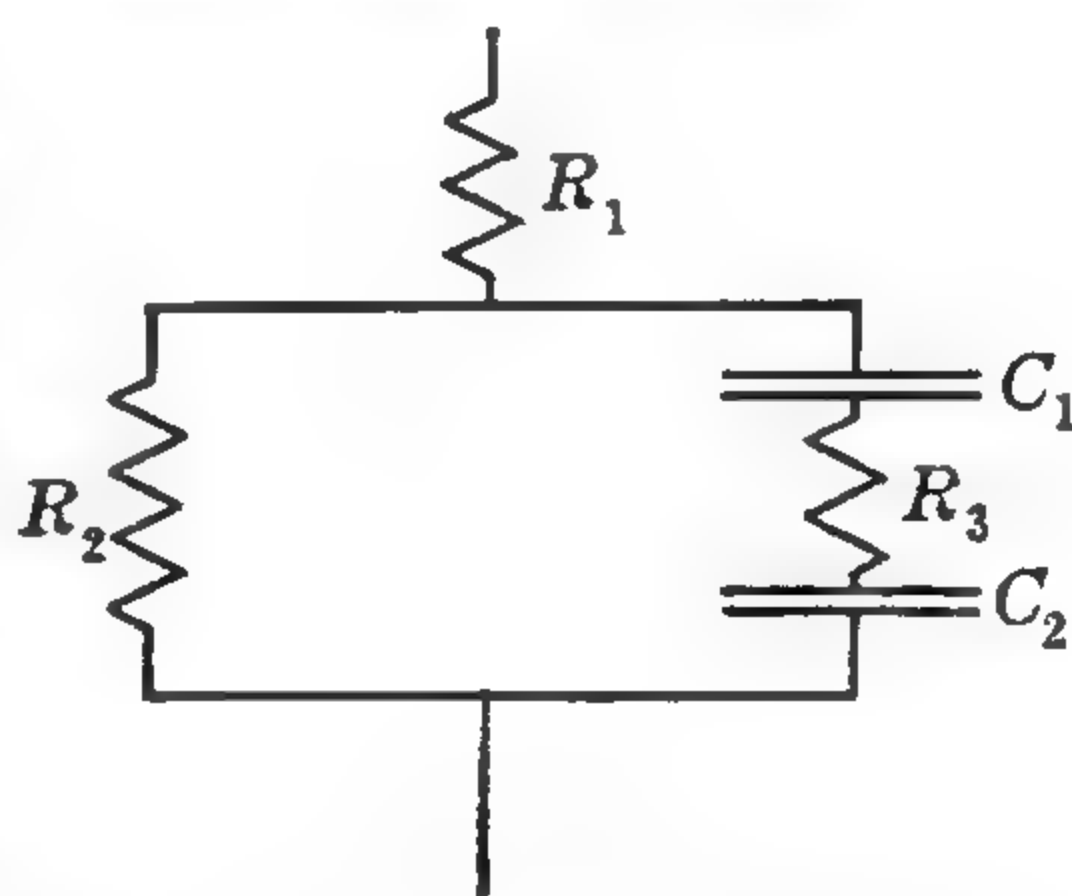


FIG. 3. Circuit equivalent to a living cell.  $R_1$  is the resistance through the medium to the cell,  $R_2$  resistance around the cell,  $R_3$  resistance inside the cell;  $C_1$  and  $C_2$  are the capacities of the cell walls.

From what has been said, it is evident that, in such mixed circuits, as the frequency is increased the reactance tends to disappear and at sufficiently high frequencies pure resistance remains. It is possible, therefore,

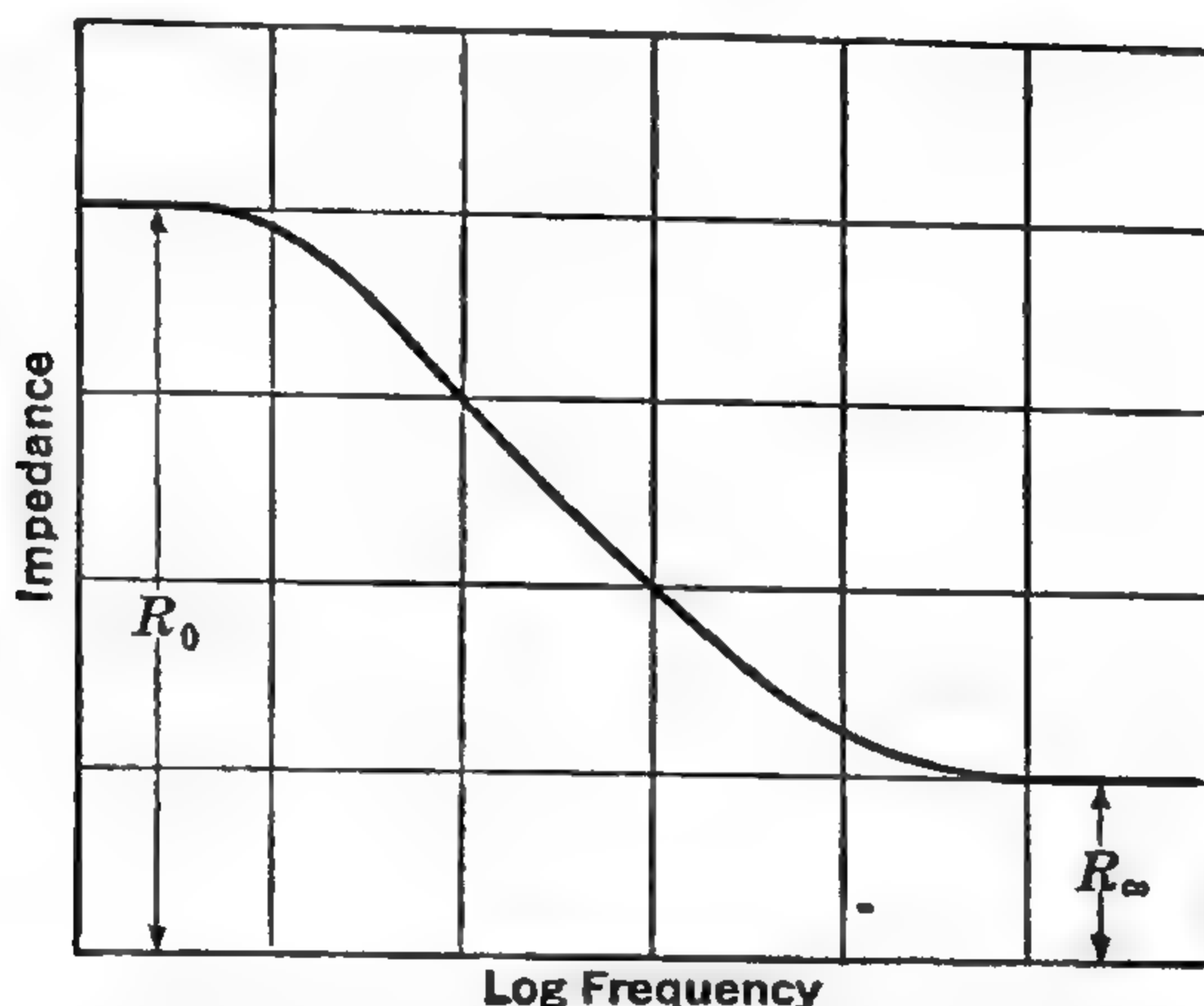


FIG. 4. Diagrammatic representation of the variation of the impedance of a biological system with frequency.

to analyze the biological circuit by varying the frequency. The general form of the variation of the impedance of biological systems is shown in Fig. 4.

By increasing the frequency sufficiently and wiping out the reactance due to the capacity of the cell wall, the conductances of the insides of the cells can be found. In general, they are surprisingly high. The interior of red blood cells, for example, has a conductance which is equivalent to that of 0.1 to 0.4 per cent KCl solutions. The reactance of living systems is due to the cell wall, which behaves as a condenser. In this connection there are two possibilities: (1) the membrane acts as a pure dielectric, or (2) the membrane shows preferential permeability for cations or for anions (the membrane becomes polarized).

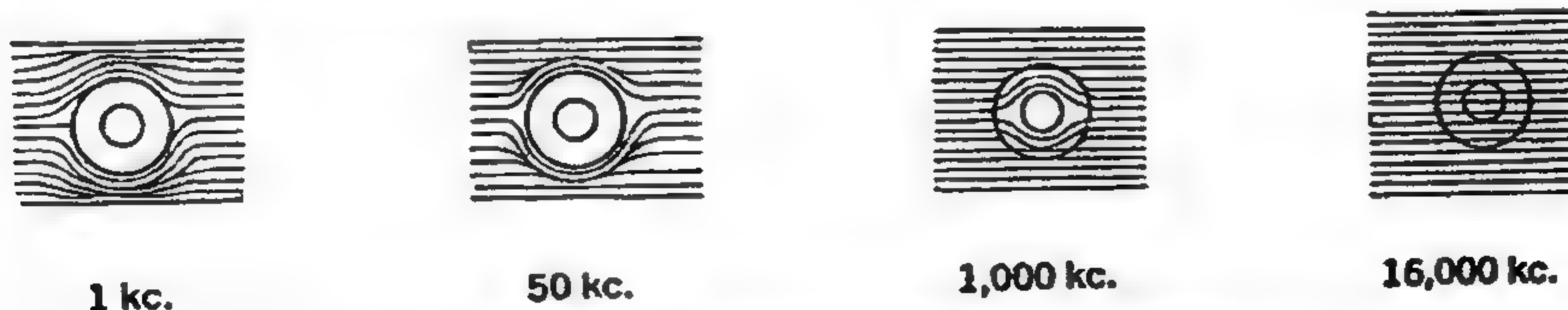


FIG. 5. Lines of current flow at the indicated frequencies through unfertilized sea-urchin eggs. (Cole.)

Figure 5 is a schematic representation of the lines of flow through suspensions of unfertilized sea-urchin eggs at the indicated frequencies.<sup>10</sup>

<sup>10</sup> K. S. Cole, *Nature* 141, 79 (1938); *Trans. Faraday Soc.* 33, 971 (1937).



Experiments indicate that living cell membranes have capacities of about one microfarad per square centimeter. The capacity of the cell membrane is largely independent of the physiological state of the cell and may be pictured as characteristic of an ion-impermeable, inert structure. The membrane resistance is very high, being in the order of  $10^8$  times that of protoplasm. When the cell is damaged or is killed, the membrane resistance decreases very radically.

Cole and Curtis<sup>11</sup> have given an excellent summary of conductance through cells.

### PROBLEMS AND QUESTIONS

1. A conductivity cell containing 0.1 molal solution of KCl at 25° C. has a resistance of 24.96 ohms. Calculate the cell constant if the specific conductance of 0.1 molal KCl is 0.012856. Filled with 0.1 molar solution of acetic acid the cell resistance is 1982 ohms. Calculate the equivalent conductance of acetic acid at this concentration.

Ans.: Cell constant = 0.3209;  $\Lambda = 1.619$ .

2. The equivalent conductance of acetic acid at 25° C. was studied with the following results:

Molar Concentration Acetic Acid	Equivalent Conductance
$2.801 \times 10^{-5}$	210.38
$1.5321 \times 10^{-4}$	112.05
$1.0283 \times 10^{-3}$	48.146
$2.4140 \times 10^{-3}$	32.217

Assume  $\Lambda_0$  equals 390.71 and obtain the ionization constant of acetic acid at infinite dilution.

Ans.:  $1.754 \times 10^{-5}$ .

<sup>11</sup> K. S. Cole and H. J. Curtis, *Medical Physics*, Vol. 2, p. 82, edited by Glasser, Year Book Publishers, Inc., Chicago, 1950.

## Chapter

# 6

## ELECTROMOTIVE FORCE CELLS

Electromotive force cells were discovered by Galvani about 1780. He found that, if a metallic conductor was placed in contact with a freshly dissected frog muscle and a second conductor in contact with the nerve which innervated the muscle, the muscle twitched as if alive when the two metallic conductors were connected. This is one of the first of many instances in which biological investigation has furthered the progress of the physical sciences.

The interest of the biochemist in electromotive force cells centers largely in two of their aspects. The first is their analytical usefulness; i.e., the activity of various electrolytes may be determined by means of the appropriate cell. The electrometric determination of hydrogen ions is an example of such a use to which cells have been put by the biochemist. The second is that they offer, on occasion, a close analogy to bioelectric potentials.

One of the oldest and simplest cells is the Daniell cell, shown in Fig. 1. It consists of a strip of zinc (zinc electrode) immersed in a solution of zinc sulfate and of a strip of copper (copper electrode) immersed in a solution of copper sulfate, the two solutions being separated by a porous diaphragm.

If the two metallic electrodes are connected by an outside wire, zinc ions are formed from the metallic zinc and copper ions deposit on the copper electrode as metallic copper. This chemical reaction leaves the zinc negatively charged and produces a positive charge on the copper. Naturally, if the two metal strips are not connected, few zinc ions can be formed from the metallic zinc and very little metallic copper will be deposited. Such a

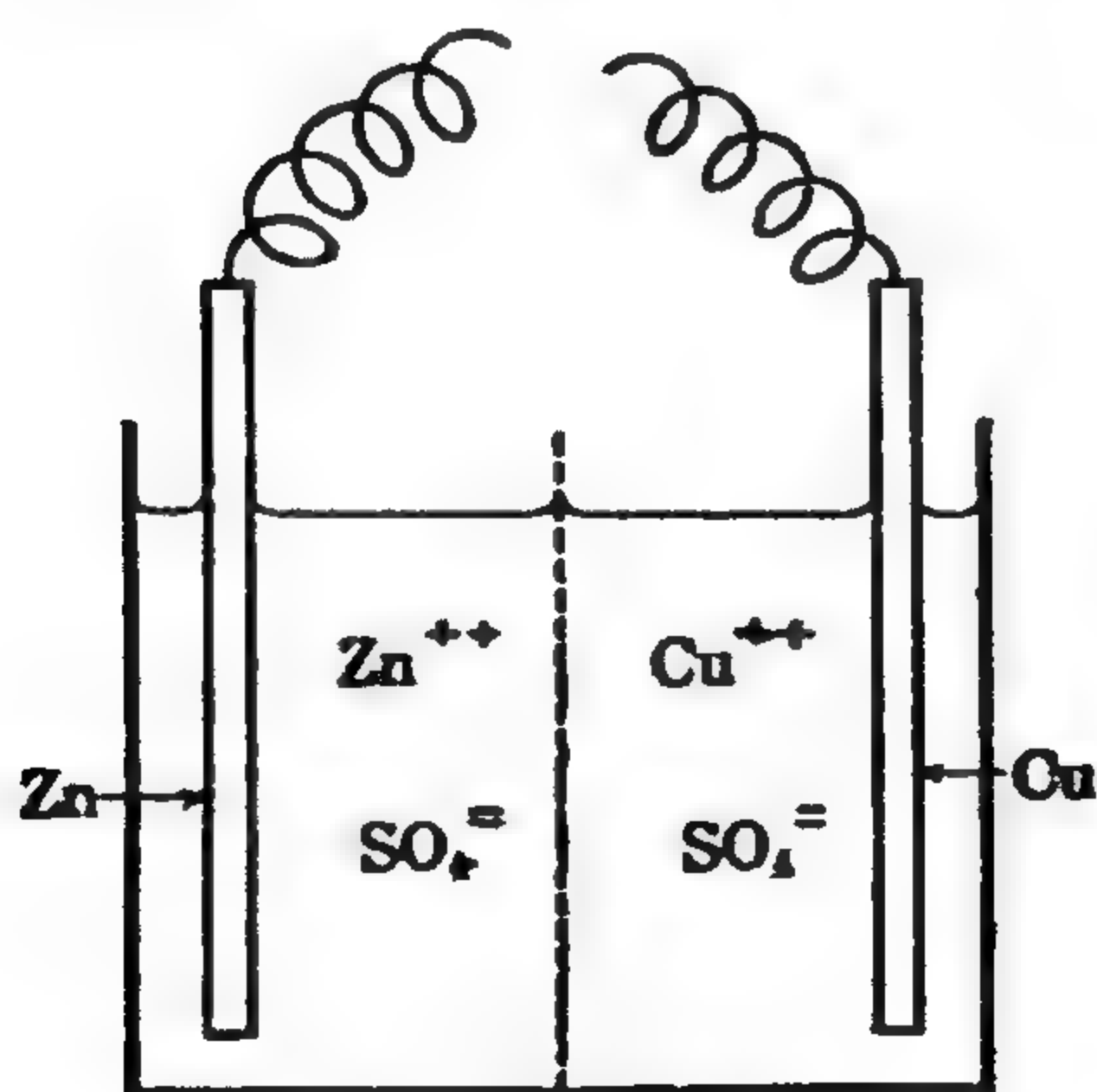


FIG. 1. Daniell cell.

process would quickly build up an electrical potential on the plates and stop the process from proceeding. When the electrodes are connected by an outside wire, the chemical reaction involved is



In order to measure the potential of such a cell an arrangement must be used that will draw little or no current from the cell; otherwise, the voltage that we measure will be too small and will not represent the voltage while the cell is performing reversibly. The measurement of the maximum voltage of a cell is usually accomplished with a potentiometer. A potentiometer

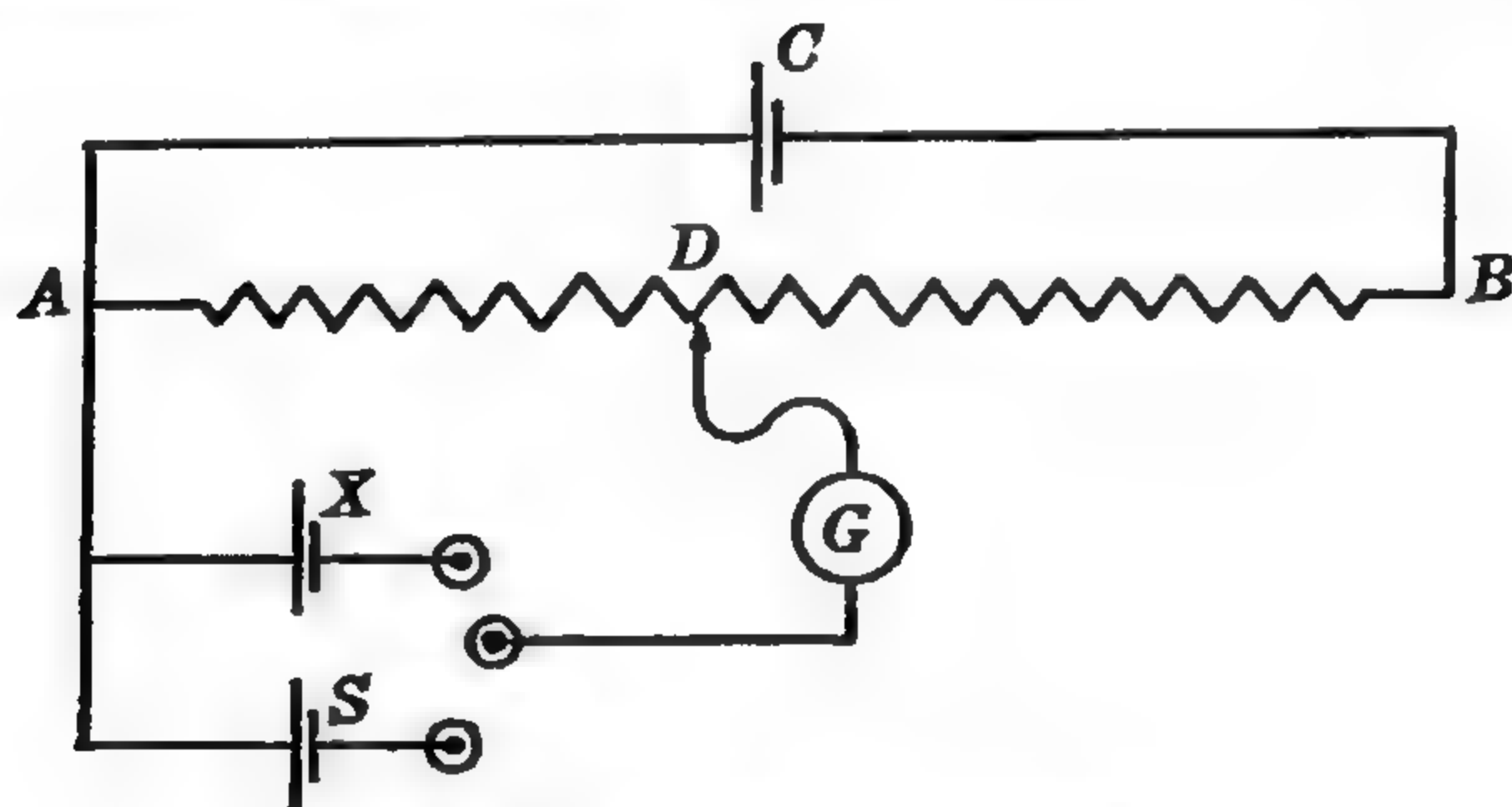


FIG. 2. Potentiometer circuit.

is an instrument that can be adjusted so that the voltage of the cell that is being measured is exactly balanced with a voltage of opposite sign, so that no current flows into or out of the cell. A diagrammatic sketch of a potentiometer circuit is shown in Fig. 2.

$AB$  is a uniform resistance wire;  $C$  is a source of constant voltage (usually a storage battery);  $D$  is a sliding contact;  $G$  is a sensitive galvanometer;  $X$  is a cell whose electromotive force is desired;  $S$  is a standard cell whose voltage is accurately known (usually a Weston cell). The slide wire  $D$  is adjusted with cell  $X$  in circuit until no current flows through the galvanometer  $G$ . The cell  $S$  is then put into the circuit, and  $D$  is again adjusted until no current flows. The electromotive force of cell  $X$  is then calculated by proportion, using the positions of  $D$  and the value of the electromotive force of cell  $S$ .

When the voltage of the galvanic cell shown in Fig. 1 is determined in the manner described, we have an accurate measure of the free energy involved in the reaction between copper ions and the zinc. The electrical work done on a mole basis is equal to the total charge transferred from the copper to the zinc multiplied by the voltage at which the transfer is made. The electrical work is exactly equal to the free energy of the process, so that

$$\Delta F = -nFE \quad (1)$$



where  $n$  is the number of equivalents involved (for the reaction pictured above  $n$  is 2), and  $F$  is the number of coulombs of electricity transferred. It has been found by experiment that to convert 1 equivalent of an element to an equivalent of ions requires 96,494 coulombs of electricity. Accordingly,  $F$  is equal to 96,494.  $E$  is the voltage that arises from the reaction between the zinc and copper ions. Other potentials are involved in the Daniell cell besides that due to the simple electronic transfer pictured above, but for the moment let us neglect these complicating voltages. When the above constants are substituted in equation 1, the free energy is obtained in joules. In order to convert to calories we must remember that 1 calorie is equal to 4.185 joules.  $\Delta F$  in calories is, therefore, given by

$$\Delta F = -23,060nE \quad (2)$$

Copper in the presence of copper ions or zinc in the presence of zinc ions or, for that matter, any element in the presence of its ions is called a half cell. Obviously it is always necessary to have two half cells connected together to realize a potential that is capable of being measured. The question arises how much of the total voltage of the Daniell cell is due to the zinc electrode and how much to the copper. The actual magnitudes of these half-cell voltages cannot be measured, but the next best thing has been done. The hydrogen half cell has been selected as the standard and is defined as having zero voltage at unit activity of hydrogen ions under 760 mm. of mercury pressure of hydrogen gas and at any temperature. We have reserved a separate chapter for the consideration of hydrogen ions and the hydrogen electrode, and this topic will be discussed in more detail at that point. The voltage of all other half cells (elements in contact with a solution of their ions) is given in reference to the standard hydrogen electrode, which, as indicated above, is defined as having zero voltage. Each element has its own normal half cell and is defined as a cell consisting of the element in its standard state in a solution of its ions at unit activity and at 25° C.

The sign of the electrode presents some minor difficulties. There are a series of conventions or rules that determine the sign to be used. The author has to confess, however, that he forgets these conventions as soon as he has learned them. One can usually guess from his knowledge of chemistry what the sign of the electrode should be; the more active metallic element tends to give off cations and, therefore, becomes negative. The sign of the electrode is always that which is actually measured by the potentiometer, and potentiometers are always stamped with the proper sign.

## SOME USEFUL HALF CELLS

Although the hydrogen half cell is the standard reference electrode, other half cells are more convenient to use. Accordingly, the potentials of these more convenient cells have been carefully compared with that of a standard hydrogen half cell and generally are used in place of the hydrogen cell. The calomel half cell and the silver-silver chloride half cells have found wide favor in this connection. The calomel half cell consists of metallic mercury in contact with a KCl solution saturated with mercurous chloride. Actually, there are three calomel half cells, known as the saturated calomel electrode, the normal calomel electrode, and the tenth normal calomel electrode. The saturated calomel half cell employs a saturated solution of KCl; the normal and the tenth normal cells contain normal and tenth normal KCl, respectively. The potentials of the saturated and of the tenth normal calomel electrodes are:

$$\text{Buffer} \parallel \text{Sat. KCl} \mid 0.1 \text{ } N \text{ KCl} \mid \text{HgCl} \mid \text{Hg} = +0.3355$$

$$\text{Buffer} \parallel \text{Sat. KCl} \mid \text{HgCl} \mid \text{Hg} = +0.2443$$

These voltages are given for 25° C. The calomel is usually connected to its companionate half cell by means of a saturated KCl salt bridge, the saturated KCl solution being held in an agar gel.

A silver-silver chloride electrode may be prepared in various ways.<sup>1</sup> One method is as follows: A platinum wire is covered with silver by electrolysis (4 volts, 1 milliampere) of a solution obtained by mixing equal volumes of 13 per cent KCN solution and 18 per cent AgNO<sub>3</sub> solution. After washing, the silver-plated platinum wire is electrolytically covered with a thin layer of AgCl, a normal solution of HCl and a current of 3.5 milliamperes being used for 20 minutes.

In addition to serving as a non-polarizable electrode in the measurement of bioelectric potentials and other potentials, the silver-silver chloride electrode can be used as a chloride electrode and the activity of a chloride-containing electrolyte can be determined with it. As we know, silver chloride is relatively insoluble; accordingly, the amount of silver ions in solution depends on the ion product constant of silver chloride, i.e.,

$$\text{Ag}^+ \times \text{Cl}^- = K_{\text{AgCl}} \quad (3)$$

Since the voltage of a silver-silver chloride electrode is given by

$$E = E_0 + \frac{RT}{F} \ln \text{Ag}^+ \quad (4)$$

<sup>1</sup> W. R. Carmody, *J. Am. Chem. Soc.* 51, 2901 (1929); 54, 188 (1932).  
M. Randall and L. E. Young, *J. Am. Chem. Soc.* 50, 989 (1928).



we can substitute relation 3 in this equation and obtain

$$E = E_0 + \frac{RT}{F} \ln \frac{K_{\text{AgCl}}}{\text{Cl}^-} \quad (5)$$

or

$$E = E_1 + \frac{RT}{F} \ln \frac{1}{\text{Cl}^-} \quad (6)$$

where  $E_1$  is a constant involving the value of the logarithm of the ion product constant of silver chloride  $K_{\text{AgCl}}$  as well as the standard electrode potential  $E_0$  of the silver-silver chloride electrode.

Half cells of sodium and of potassium have been employed. The chemical reactivity of these elements necessitates the use of sodium or of potassium amalgam since the naked elements quickly react with water and spoil the electrode. Actually, the amalgam electrodes have a very limited biological application.

Calcium ions play very important biological roles, and it would greatly facilitate research on calcium and calcium ions if a proper calcium electrode could be devised. Calcium, like sodium and potassium, is so active that a calcium amalgam must be used in place of metallic calcium. Joseph<sup>2</sup> has devised a calcium electrode in which the calcium amalgam is separated from the solution under investigation by a cellophane membrane. Such an electrode seems to work as long as the solutions investigated contain no diffusible material which reacts with the amalgam. This condition is a serious objection, however, severely limiting the usefulness of such an electrode.

Joseph<sup>3</sup> has proposed the use of lead amalgam in contact with lead oxalate and calcium oxalate. This electrode involves the solubility-product constant of both lead oxalate and calcium oxalate. The equation for this cell is

$$E = E_0 - \frac{3RT}{2F} \ln A_{\text{CaCl}_2} \quad (7)$$

where the mean activity of  $\text{CaCl}_2$  is

$$A_{\text{CaCl}_2} = \sqrt[3]{A_{\text{Ca}^{++}} \times A_{\text{Cl}^-}^2} \quad (8)$$

This electrode has two requirements: (1) there must not be anions other than  $\text{C}_2\text{O}_4^{--}$  which form insoluble lead salts, and (2) there must not be other cations than calcium which form insoluble oxalates.

<sup>2</sup> N. R. Joseph, *J. Biol. Chem.* 126, 389 (1938).

<sup>3</sup> N. R. Joseph, *J. Biol. Chem.* 130, 203 (1939).



McLean and Hastings<sup>4</sup> have developed a biological method for the estimation of calcium ions. The method consists essentially in direct comparison and matching of known solutions with biological fluids of unknown calcium-ion concentration, the criterion for equal calcium-ion concentration being the production of equal amplitude of contraction of the ventricle of the isolated heart of the frog. The contractions are recorded on a rotating smoked drum.

Actually, the calcium which is diffusible through a collodion membrane of the proper porosity is probably almost entirely in the ionic form: accordingly, the calcium-ion concentration can be fairly closely estimated in a biological fluid by ultrafiltration through a properly prepared collodion membrane followed by a calcium determination of the ultrafiltrate.

It must be emphasized that in measuring potentials in any solution, biological or otherwise, non-polarizable electrodes are required. For example, suppose that one wished to measure the potential across a frog's skin which is bathed on both sides by KCl solutions. On first thought it might seem sufficient to plunge two platinum wires into the two KCl solutions separated by the frog's skin and measure the potential directly. This, however, is not possible. A potential difference will be observed between the platinum wires, but it will be very erratic and bear little relation to the actual potential difference between the two solutions. The proper technique is to place two silver-silver chloride electrodes in the KCl solutions, or connect the two solutions by salt bridges to two identical calomel electrodes, and measure the difference in potential between the non-polarizable electrodes. The reason why the platinum wires will not and the non-polarizable electrodes will yield the true potential difference should be clear. The platinum wires provide no mechanism for the flow of electricity from the metal to the solution or from the solution to the metal. There is no possibility of platinum ions being formed from the wire or for the deposition of ions from the solution on the wire as metallic potassium or as chlorine gas. Such an electrode is quickly polarized.

### CONCENTRATION CELLS

If, for the copper electrode in the Daniell cell, Fig. 1, we substitute another zinc electrode, and for the  $\text{CuSO}_4$  solution a solution of  $\text{ZnSO}_4$  at a concentration different from that in compartment 1, Fig. 1, we have what is known as a concentration cell. A potential difference can be observed between the two zinc electrodes. It is easy to see, in a qualitative manner, how such a potential could and would arise. Let us suppose that the  $\text{ZnSO}_4$  solution in compartment 1 is more concentrated than that in compartment 2. Under these circumstances the tendency for zinc ions to be formed from the metallic

<sup>4</sup> F. C. McLean and A. B. Hastings, *J. Biol. Chem.* 107, 337 (1934).

zinc would be less in compartment 1 than in compartment 2, and therefore the zinc in compartment 1 would be positive to the zinc in compartment 2. As we have seen in Chapter 2 the free energy experienced in diluting a solution from activity 1 to activity 2 is

$$\Delta F = RT \ln \frac{A_2}{A_1} \quad (9)$$

and since

$$\Delta F = -nFE \quad (10)$$

we have

$$E = -\frac{RT}{nF} \ln \frac{A_2}{A_1} \quad (11)$$

and at 25° C.

$$E = -\frac{2.3026 \times 8.3145 \times 298.15}{96,494n} \log \frac{A_2}{A_1} \quad (12)$$

$$E = -\frac{0.059}{n} \log \frac{A_2}{A_1} \quad (13)$$

Knowing the activity of zinc sulfate in either compartment, we can, after measuring the potential difference between the zinc electrodes, calculate the activity of the zinc sulfate in the other compartment. It should be noted, however, that what we measure in this, as well as in other electromotive force cells, is not the individual ionic activities but a mean activity of the positive and negative ions. That is,

$$\text{Mean activity} = \sqrt{A^+ \times A^-} \quad (14)$$

It is impossible at the present time to measure individual ion activities.

The arrangement pictured above of two zinc-zinc sulfate half cells connected together is known as a concentration cell; in fact, all cells that include two half cells of the same element but at different ionic concentrations are known as concentration cells.

### LIQUID JUNCTION POTENTIAL

The potential differences that we observe in concentration cells such as pictured above are not exactly given by equation 11. We have in addition a potential difference at the junction of the two liquids which is technically known as a liquid junction potential. The liquid junction potential is essentially due to differences in ionic mobility. For example, in the concentration cell involving zinc sulfate, the zinc sulfate from the more-concentrated solution diffuses into the more-dilute solution. The mobility of the sulfate ion is about one and one-half times greater than that of the zinc ion; the result is that the concentrated zinc sulfate solution tends to be positive in respect to the more-dilute solution.

Henderson<sup>5</sup> derived an equation that expresses the liquid junction potential as a function of the concentration and mobility of the ions.

If the two solutions contain a single univalent salt at two concentrations  $C_1$  and  $C_2$ , Henderson's equation reduces to

$$E_L = \frac{RT}{F} \frac{u - v}{u + v} \ln \frac{C_1}{C_2} \quad (15)$$

where  $u$  and  $v$  are the mobilities of cation and anion, respectively.

Naturally, if  $u$  equals  $v$  the liquid junction potential becomes zero. This condition is nearly realized with solutions of KCl, and it is the basis for the use of KCl in salt bridges connecting half cells. This is not an entirely unambiguous procedure, but it does represent the best, under certain circumstances, that can be done.

### CELLS WITHOUT LIQUID JUNCTIONS

Although, as we have seen, liquid junction potentials have an inherent interest, they introduce uncertainties in electromotive force measurements which are disturbing. The difficulty of the liquid junction can be circumvented by making a cell without a liquid junction. For example, Nims and Smith<sup>6</sup> used the cell shown in Fig. 3.

Because of the very low solubilities of hydrogen and of silver chloride, the solution between the electrodes is practically of uniform composition, even though the hydrogen and the silver chloride must be kept in separate parts of the containing vessel. Such a cell is known as a cell without a liquid junction.

If we have two such cells as are pictured in Fig. 3 containing HCl at different concentrations, the difference in their voltages will evidently be the voltage of a true concentration cell, and we have

$$E_2 - E_1 = E = \frac{2RT}{F} \ln \frac{A_2}{A_1} \quad (16)$$

where  $A_2$  and  $A_1$  are activities of HCl in two cells.

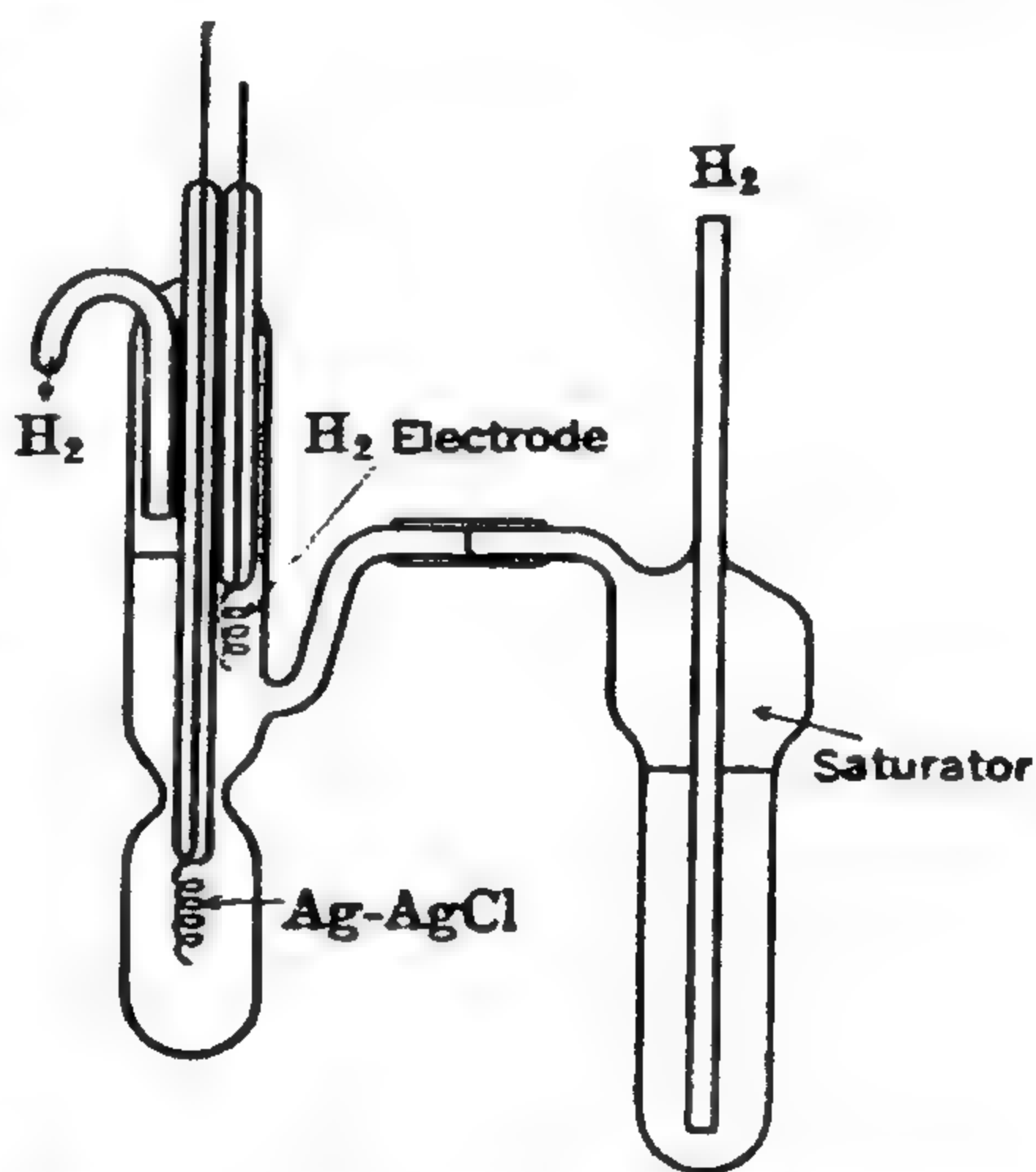


FIG. 3. Cell without liquid junctions. (Nims and Smith.)

<sup>5</sup> P. Henderson, *Z. Physik. Chem.* 59, 118 (1907).

<sup>6</sup> L. F. Nims and P. K. Smith, *J. Biol. Chem.* 113, 146 (1936).



This is the type of measurement which is meant when reference is made to a concentration cell without a liquid junction. The distinctive feature of a cell without liquid junction is that two types of electrodes are involved, each of which must be reversible to an ion constituent in the solution contained in the cells.

### MEMBRANE POTENTIALS

The liquid junction potential can be greatly increased if the two electrolyte solutions are separated by a very dense membrane with small pore size, such as well-dried collodion membranes. Michaelis<sup>7</sup> found that, when 0.01 *M* KCl was separated from 0.1 *M* KCl solution by such a well-dried collodion membrane, a potential difference was established across the membrane which amounted, when the membrane pore size was very small, to 45 to 55 millivolts. The dilute solution was always positive in respect to the concentrated solution. Evidently, the rate of diffusion of the anion had been greatly diminished in comparison with that of the cation.

Applying the formula for the liquid junction potential to the extreme situation where the collodion membrane is completely impermeable to the chloride ion, we have

$$E_L = - \frac{u}{u} \frac{RT}{F} \ln \frac{C_2}{C_1} \quad (17)$$

and at 25° C.

$$E_L = -0.059 \log \frac{C_2}{C_1} \quad (18)$$

When  $C_2$  is ten times greater than  $C_1$ ,  $E_L$  will be 59 millivolts. It can be seen that the more dense collodion membranes of Michaelis approached closely the theoretical impermeability to anions. Such potentials under favorable conditions were maintained for long periods of time (several months), but eventually the potentials approach zero.

Much biochemical work has been done on membranes of the collodion type. Elford<sup>8</sup> has given directions for preparing what he calls gradocol membranes. Such membranes have reproducible characteristics and are, within limits, as dense or open as may be desired. The principle of their preparation depends on the addition of the proper solvent to the collodion previous to the formation of the membrane. Elford found that, in general, the addition of good solvents for nitrocellulose (ordinary collodion is a solution of nitrocellulose in an ether-alcohol mixture) caused a decrease in membrane porosity, and non-solvents or precipitating agents an increase in porosity. It was possible to compose mixtures of ether, ethyl alcohol, amyl alcohol, and acetone to which were added small quantities of other

<sup>7</sup> I. Michaelis, *Colloid Symposium Monograph* 5, 135 (1928); *Koll. Z.* 62, 21 (1935).

<sup>8</sup> W. I. Elford, *Trans. Faraday Soc.* 33, 1094 (1937); *J. Path. Bact.* 34, 505 (1931).

reagents for the preparation of membranes covering a wide range of porosities ( $2\ \mu$  to  $2\ m\mu$ ). Fine adjustments in porosities were made by changing the evaporation time of the solvents. The membranes were formed by pouring a thin layer of the collodion solution on a carefully prepared glass plate and allowing evaporation of the solvents to proceed for a specified time. The film was then covered with water and the solvents washed out. Collodion sacks can be formed inside test tubes; the sacks, after the solvents have evaporated, are treated with water and carefully withdrawn from the test tube. Sacks, however, are unsuited for work requiring uniform and reproducible membranes; disk membranes must be used in such work.

Sollner<sup>9</sup> and co-workers have studied the electrical behavior of collodion membranes in much detail. They concluded that the differential permeability to ions shown by the collodion membranes was due to acid groups (carboxyl groups) in the collodion and it was quite impossible for them to achieve significant membrane potentials with the purer grades of collodion on the market. If, however, the collodion membrane was oxidized by treatment with sodium hydroxide, the membranes acquired pronounced electrochemical properties. Membranes of this kind are negatively charged and restrict the movement of anions. Positively charged collodion membranes could be prepared by immersing collodion membranes in solutions of protamine chloride or sulfate buffered to pH about 10.5. The protamine was adsorbed on the membrane, and, after several days contact with the protamine solution, the membranes were washed and ready for use. Protamines are basic proteins with isoelectric point about pH 12 and with a molecular weight of about 3000.

The transport of cations through a negatively charged membrane is an example of base exchange. The metal cation exchanges for the hydrogen ion attached to the carboxyl group, and it is, accordingly, possible for the metal cation to penetrate the membrane. Those membranes with a high base exchange value always have great electrochemical activity. The base exchange capacity is measured by titrating an acidified membrane with standard base as with any acid-base titration.

Sollner points out, however, that the structure of a collodion membrane must have a very heterogeneous character; a good physical picture, much enlarged, is presented by a wad of cotton. Many of the carboxyl groups responsible for base exchange cannot participate in the transport of cations or in the repulsion of anions because they are not placed strategically in the pores of the membrane. It is to this fact that Sollner attributes the relative quantitative failure of the theories of Teorell<sup>10</sup> and of Meyer and Sievers.<sup>11</sup>

<sup>9</sup> K. Sollner, *J. Phys. Chem.* **49**, 47, 171, 265 (1945).

<sup>10</sup> T. Teorell, *Proc. Natl. Acad. Sci. U. S.* **21**, 152 (1935).

<sup>11</sup> K. H. Meyer and J. F. Sievers, *Trans. Faraday Soc.* **33**, 1073 (1937).



who sought to express the electrochemical behavior of membranes in terms of the base exchange capacity of the membranes.

Differential ion permeability gives rise to what is known as an anomalous osmosis. It has been found that under certain conditions water will flow from a concentrated to dilute salt solution. The two solutions are separated by a porous membrane. The flow may continue for several hours and build up a considerable pressure. After a time, however, the pressure tending to force the water from the concentrated to the dilute solution falls to zero and the electrolyte concentration on both sides of the membrane becomes identical. Anomalous osmosis is a kinetic and not an equilibrium condition. If the water flows from the dilute to the concentrated salt solution, the process is called positive anomalous osmosis; if the direction of flow is from the concentrated to the dilute solution, it is called negative anomalous osmosis.

Anomalous osmosis is clearly the result of the membrane potentials which we have discussed above. Such potentials arise, as we have seen, from a differential permeability of the membrane to anions and cations. Since the potentials produce an electroosmotic flow of water (see Chapter 9) across the membrane, the direction of flow depends on the sign of the diffusion potential, which in turn depends on the relative mobility of the anions and cations through the membrane.

The conditions for negative anomalous osmosis may be outlined as follows: If the ion whose sign of charge is the same as that of the membrane has the greater mobility through the membrane, negative anomalous osmosis will occur. In order for this condition to be fulfilled, the pores of the membrane must be fairly large and an electrolyte must be used whose ions show considerable difference in mobility, the greater mobility belonging to the ion whose sign of charge is the same as that of the membrane. A specific example of anomalous negative osmosis is presented by a solution of  $\text{LiCl}$  or  $\text{LiNO}_3$  and a magnesium silicate membrane. The mobility of  $\text{Li}^+$  ions is much less than the mobilities of either  $\text{NO}_3^-$  or  $\text{Cl}^-$  ions. (See page 98 Table 1. In spite of the negative charge on the magnesium silicate, the pores of this membrane are so large that the mobility of the anion through it is not significantly diminished and, accordingly, the anions move through the membrane faster than the cations. Consequently the water side of the membrane becomes negatively charged in respect to the salt side. The water being positively charged in the negative pores is drawn from the salt side to the water side by electroosmosis, and negative anomalous osmosis results.

On the other hand, positive anomalous osmosis will occur if the pores of the membrane are very small. In such a situation the ions whose sign of charge is opposite to that of the membrane pores will penetrate faster.



This will give rise to a charge on the salt side of the membrane which has the same sign as that on the membrane and opposite to that of the water contained in the pores of the membrane. This will result in an electro-osmotic flow of water from the dilute to the concentrated side of the membrane, i.e., positive anomalous osmosis. Naturally, the pore size of a negatively charged membrane need not be small if the cation initially has a greater mobility than the anion; all acids fulfill this condition.

If two equimolar solutions of electrolytes contain univalent cations and a common anion, the diffusion potential becomes at 25° C.

$$E = 0.059 \log \frac{u_1 + v}{u_2 + v} \quad (19)$$

If the mobility of the anion through the membrane be reduced to zero, the potential observed across the membrane then becomes a function only of the relative rate of penetration of the two cations. Such potentials are known as bi-ionic potentials.<sup>12</sup> The relative rate of mobility of the cations across a membrane is a function of several complicated factors such as the relative adsorbability of the cations as well as their relative sizes.

## BIOELECTRIC POTENTIALS

The subject of bioelectric potentials is an important one for the physiologist and the biochemist. These potentials undoubtedly arise from a variety of causes, among which may be mentioned: (1) membrane potentials due to a Donnan equilibrium; (2) diffusion potentials, i.e., liquid junction potentials; (3) electrokinetic potentials due to motion of charged particles or fluids; and (4) phase potentials resulting from contact between a solid and liquid phase, or between two liquid phases, or between two solid phases. The potentials resulting from Donnan equilibria and from motion of charged particles or of fluids will be dealt with in later portions of this book. At the moment, let us consider the potentials arising from diffusion and from phases in contact.

The ionic composition of a living cell differs, in general, considerably from that of the surrounding fluids. It is true that living cells are in osmotic equilibrium with fluids in contact with them, but the distribution of individual ions is usually far from equal. This difference in ionic composition is maintained either through constant metabolism on the part of the cell or through selective impermeability of the cell membrane. The mammalian red cell, for example, shows a very limited permeability to cations. If a living cell is injured so that the membrane is destroyed or the metabolism of the cell interrupted, the electrolytes diffuse into or out of the cell.

<sup>12</sup> K. Sollner, *J. Phys. Colloid Chem.* 53, 1211, 1226 (1949).

depending on the direction of the concentration gradients. This diffusion of ions gives rise to the injury potentials. It has been found that injured tissue is almost always negative to the uninjured tissue, although positive injury potentials can be obtained if the solution which bathes the tissue has the required composition.

Steinbach<sup>13</sup> has investigated injury potentials of the adductor muscle of the common scallop. In his experiments, he exposed a cross section of the adductor muscle and measured the difference in potential between the injured and uninjured parts. The experiments were carried out by placing the tip of a silver-silver chloride electrode in the approximate center of the cut surface of the muscle and the other silver-silver chloride electrode on the shell at the base of the muscle cylinder. Injury potentials of 30 to 40 millivolts were found. These potentials decreased with time until in about an hour they had disappeared.

The effects of various electrolytes in contact with the injured portion were studied. Calcium chloride solutions greatly decreased the time of recovery, whereas potassium chloride solutions prolonged it. Sodium and magnesium ions were intermediate in effect between potassium and calcium. It is believed that the reason why calcium speeded recovery is that calcium ions bring about precipitation or coagulation of a membrane which protects the injured tissue. Later Steinbach<sup>14</sup> studied diffusion potentials across uninjured scallop adductor muscle. In this instance the muscle was bathed in the electrolyte solution under investigation, and the complete electromotive force cell which he employed was



By assuming an anion in the tissue of low mobility he was able to use the Henderson liquid junction equation to predict the observed potentials.

The observed potentials of the scallop muscle were considerably higher than could arise from the free diffusion of ordinary, inorganic electrolytes. As indicated above, Steinbach had to assume the presence of a slow-moving anion in the tissue. He believes this anion to be protein, and he studied the diffusion potentials resulting from diffusion of ions in gelatin gels. In this system he was able to show a behavior that closely paralleled that of the scallop muscle. He came to the conclusion that diffusion potentials can account quantitatively for injury potentials provided that the slow-moving anion of the tissue is considered.

Action currents are closely related to, if not identical in origin with, injury potentials. Action currents are associated with any stimulation of

<sup>13</sup> H. B. Steinbach, *J. Cellular Comp. Physiol.* **3**, 203 (1933).

<sup>14</sup> H. B. Steinbach, *J. Cellular Comp. Physiol.* **7**, 291 (1935).



protoplasm. Heart waves as observed in an electrocardiogram are an example of such action currents. The most elegant and dramatic example of an action current is that produced by an electric eel. It has been suggested that action currents arise as a result of a polarization of the cell membrane. This polarization results from a differential permeability of the membrane to ions. Upon stimulation the differential permeability is momentarily lost and a flow of ions across the membrane results; a flow of current is thereby produced. An excellent summary of excitation and propagation is given by Curtis and Cole<sup>15</sup> in which they discuss action potentials and related subjects.

### PHASE POTENTIALS

When two phases are brought into contact a difference of potential between the phases results. Two metals in contact, for example, will give rise to a potential difference; this is the basis of the thermocouple which is used for temperature measurements, the potential difference being a function of the temperature. The two metal phases show a different "solubility" for electrons. We shall see in the chapter on electrokinetics that phase potentials are of a very general occurrence and arise not only with metals in contact but also with non-metals and can involve solids, liquids, and gases. The difference in potential may be due to a flow of ions from one phase to another. Since in living tissue there are many phase separations (interfaces), such potentials are of common physiological occurrence. There are appropriate techniques for measuring such potentials, as we shall see in the chapter on electrokinetics. The question arises whether or not they can be measured by any of the techniques that we have outlined in the present chapter.

There have been a great many measurements of the electromotive cell of the type



in which the salt solutions contain the same electrolyte at different concentrations or they may contain different salts. By "oil" is meant some substance which is immiscible with water. Investigations along this line led to the discovery of the glass electrode.<sup>16</sup> Beutner has published a monograph on such measurements.<sup>17</sup> He felt that the potential difference observed in such an oil electrode results from a "solubility" of the ions in the

<sup>15</sup> H. J. Curtis and K. S. Cole, *Medical Physics*, Vol. 2, p. 584, edited by Glasser, Year Book Publishers, Chicago, 1950.

<sup>16</sup> M. Cremer, *Biochem. Z.* 47, 562 (1906).

<sup>17</sup> R. Beutner, *Die Entstehung elektrischer Ströme in lebenden Geweben*, Verlag von Ferdinand Enke, Stuttgart, 1920.



oil. He emphasized the distribution coefficient of the ions between the oil and water phases. As it is not possible to obtain independent evidence for such a distribution, Beutner's theory cannot be put to test. Beutner attempted to explain membrane potentials by analogy with the oil potentials, but the connection is far from clear.

### PROBLEMS AND QUESTIONS

1. Two hydrogen electrodes are connected by a 0.1 *M* KCl salt bridge. One of the electrodes is in 0.005 *m* HCl and the other 0.10 *m* HCl. Use Table 2 of Chapter 4, to find the standard potential and calculate the e.m.f. which should be observed at 25°C. *Ans.*: 0.0167 volt.
2. Calculate the liquid junction potential at 25°C. between two solutions of NaCl. One solution is at 0.001 *m* and the other at 0.01 *m*. Use Table 1, Chapter 5 to find the transport numbers of ions. Make a similar calculation for KCl. *Ans.*: 0.0122 volt for NaCl, 0.0012 volt for KCl.
3. What are the conditions for the appearance of liquid-junction potentials?

## ACIDS and BASES

According to Samuel Johnson,<sup>1</sup> acids are composed of pointed particles which affect the taste in a sharp and piercing manner. Our present ideas concerning the nature of acids have become somewhat more precise than Doctor Johnson's, although there are no less than five competing theories of acids and bases which are current.<sup>2</sup>

The theory proposed independently by Brønsted<sup>3</sup> and by Lowry<sup>4</sup> seems adequate for our needs. This theory conceives of acids as substances that are capable of yielding protons, the proton being the unhydrated hydrogen ion. A base, on the other hand, is defined as a substance that is able to unite with protons, i.e., a proton acceptor. Their theory may be expressed by the following equation



where  $A$  is an acid and  $B$  is a base. The reaction of acids and bases, accordingly, need not involve the elements of water. For example,



is clearly analogous to



The sodium acetate acts as a base just as does sodium hydroxide.

<sup>1</sup> Samuel Johnson, *Dictionary*, Fourth Edition, W. Strahan, London, 1773.

<sup>2</sup> W. F. Loder and S. Zuffanti, *Electronic Theory of Acids and Bases*, John Wiley & Sons, New York, 1947.

<sup>3</sup> J. N. Brønsted, *Rec. trav. chim.* 42, 718 (1923); *Chem. Revs.* 5, 231 (1928).

<sup>4</sup> T. M. Lowry, *Chemistry & Industry* 42, 43 (1923).

## IONIZATION OF WATER

We know that water dissociates into hydrogen and hydroxyl ions, i.e.,



which can be expressed as

$$K_A = \frac{A_{\text{H}^+} \times A_{\text{OH}^-}}{A_{\text{H}_2\text{O}}} \quad (1)$$

where  $A_{\text{H}^+}$ ,  $A_{\text{OH}^-}$ , and  $A_{\text{H}_2\text{O}}$  denote the activities of the hydrogen ions, hydroxyl ions, and water molecules, respectively. Since in any ordinary aqueous solution the activity of water is so much greater than that of either the hydrogen or hydroxyl ions, we may assume that its activity is constant, or

$$A_{\text{H}^+} \times A_{\text{OH}^-} = K_{\text{WA}} \quad (2)$$

where  $K_{\text{WA}}$  is a constant which involves the dissociation constant of water as well as the activity of water.

The ionization constant of water has been accurately studied by Harned and Hamer,<sup>5</sup> using the appropriate electromotive force cell. Their results, given in Table 1, show how the ion activity product constant of water varies with the temperature.

TABLE 1

$K_{\text{WA}}$  AS A FUNCTION OF TEMPERATURE (HARNED AND HAMER)

Temperature, °C.	$K_{\text{WA}} \times 10^{14}$
0	0.115
10	0.293
20	0.681
25	1.008
30	1.471
40	2.916
50	5.476
60	9.614

## IONIZATION OF WEAK ACIDS

Some acids and bases are completely dissociated, such as, for example, the first hydrogen of sulfuric, nitric, and hydrochloric acids, and sodium, potassium, and the alkali-earth hydroxides. We have already discussed in the chapter entitled "Ions in Solution" electrolytes which are completely ionized. Here we are interested in the so-called weak acids and bases, i.e., acids and bases that do not ionize completely.

<sup>5</sup> H. S. Harned and W. J. Hamer, *J. Am. Chem. Soc.* 55, 2194 (1933).



Consider a weak acid such as acetic acid dissolved in water



It will be evident that, though the above dissociation can be expressed in terms of an equilibrium constant, two such constants will be obtained, depending on whether we use ionic concentrations or ionic activities. Thus, using concentrations, we have

$$K_c = \frac{C_{\text{H}^+} \times C_{\text{A}^-}}{C_{\text{HA}}} \quad (3)$$

or using activities

$$K_A = \frac{A_{\text{H}^+} \times A_{\text{A}^-}}{A_{\text{HA}}} \quad (4)$$

The relation between these constants is given by

$$K_A = \frac{\gamma_{\text{H}^+} \gamma_{\text{A}^-}}{\gamma_{\text{HA}}} \cdot K_c \quad (5)$$

where  $\gamma_{\text{H}^+}$ ,  $\gamma_{\text{A}^-}$ , and  $\gamma_{\text{HA}}$  are the activity coefficients of the hydrogen ions, anions, and undissociated acid, respectively. The value of  $K_A$  is independent of electrolyte concentration; that of  $K_c$  will vary with electrolyte concentration.  $K_A$  is called the thermodynamic or true acid dissociation constant, and  $K_c$  is called the stoichiometric constant. It is evident that  $K_A$  is by far the more useful and fundamental constant. Not infrequently, a confusion arises as to which constant is meant, and tables of constants do not always distinguish one from the other.

The two methods most frequently used to evaluate acid and base ionization constants are (1) conductance measurement and (2) the electromotive force method. The older measurements were made almost exclusively through conductance determinations. As originally employed, this method does not yield true dissociation constants. With modern refinements in calculations, however, this method is capable of giving quite as accurate results as the electromotive force method.

The conductance method depends on the measurement of the conductance of a series of dilutions of a pure acid. The equivalent conductance is determined, and, since the conductance due to each ion is known, the ion product can be calculated. The technique of the calculation of the true dissociation constant from such data is described by MacInnes<sup>6</sup> and can be summarized as follows: As we have seen, for a weak acid

$$K_A = \frac{\gamma_{\text{H}^+} \gamma_{\text{A}^-}}{\gamma_{\text{HA}}} \cdot K_c \quad (6)$$

<sup>6</sup> D. A. MacInnes, *Cold Spring Harbor Symposia Quant. Biol.* 1, 190 (1931).

The activity coefficient of the anion is assumed equal to that of the hydrogen ion. The activity coefficient of the uncharged, unionized acetic acid does not depart significantly from unity. We can, therefore, write equation 6 as

$$K_A = \gamma_{H^+}^2 K_c \quad (7)$$

and, taking the logarithm of both sides,

$$\log K_A = \log K_c + 2 \log \gamma_{H^+} \quad (8)$$

From the Debye-Hückel theory, at 25° C. and at low ionic strengths,

$$-\log \gamma = 0.506 \sqrt{C} \quad (9)$$

Substituting 9 in 8 leads to

$$\log K_A = \log K_c - 1.012 \sqrt{C} \quad (10)$$

The value of  $K_c$  is determined by conductance measurements for a series of dilutions, and  $\log K_c$ , thus obtained, is plotted against  $\sqrt{C}$ .

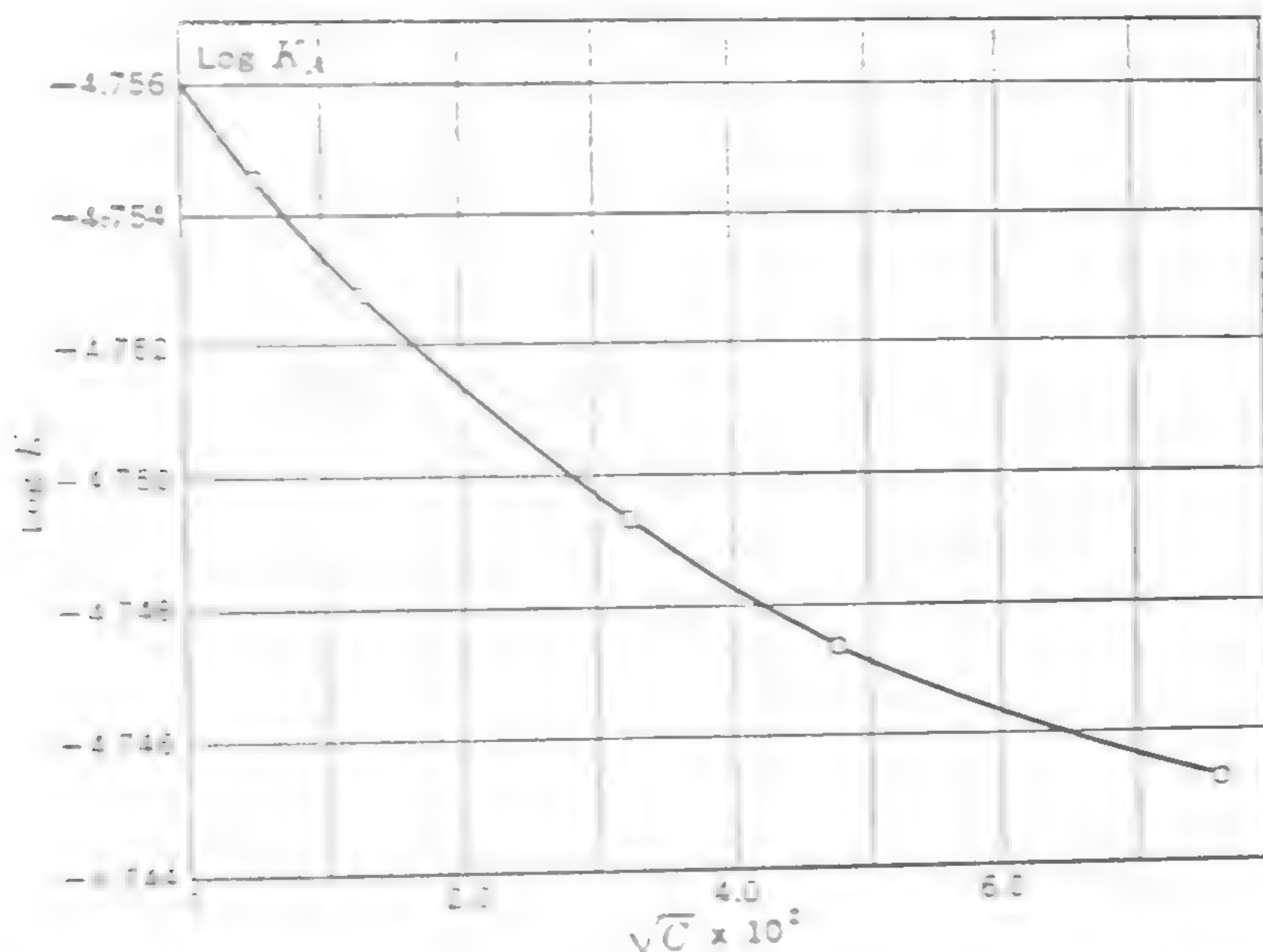


FIG. 1. Plot of  $\log K_c$  obtained from conductance measurements against the square root of the acetic acid concentration.

Evidently, when  $C$  is zero,  $\log K_c$  must equal  $\log K_A$ . The intercept on the  $y$ -axis is, therefore, equal to  $\log K_A$ . Such a plot for acetic acid is shown

The electromotive force method for acid dissociation constants was first formulated by Harned and Ehlers.<sup>7</sup> They employed a cell without a liquid junction which consisted of the following



The equation for this cell is

$$E = E_0 - \frac{RT}{F} \ln \gamma_{\text{H}^+} \gamma_{\text{Cl}^-} m_{\text{H}^+} m_{\text{Cl}^-} \quad (11)$$

where  $m_{\text{H}^+}$  and  $m_{\text{Cl}^-}$  are the molalities of the hydrogen and the chloride ions, respectively.  $E_0$  is the normal electrode potential of the cell



Substituting in equation 11 the expression for the thermodynamic dissociation constant of a weak acid, and rearranging, there results

$$E - E_0 + \frac{RT}{F} \ln \frac{m_{\text{HA}} m_{\text{Cl}^-}}{m_{\text{A}^-}} = - \frac{RT}{F} \ln \frac{\gamma_{\text{H}^+} \gamma_{\text{Cl}^-}}{\gamma_{\text{H}^+} \gamma_{\text{A}^-}} - \frac{RT}{F} \ln K_{\text{A}} \quad (12)$$

The first member of the right side of equation 12 becomes zero at infinite dilution; the activity coefficients approach unity as the concentration decreases. Accordingly, if the left side of equation 12 is plotted against the ionic strength, a very nearly straight line is obtained which can be extrapolated to zero ionic strength. The intercept at zero ionic strength is  $-\frac{RT}{F} \ln K_{\text{A}}$ , from which  $K_{\text{A}}$  can be computed.

The only unknown in the above is  $E_0$ , and this can be evaluated by a method suggested by Hitchcock.<sup>8</sup> The equation for the cell of which  $E_0$  is the normal potential is

$$E_0 = E + \frac{4.6RT}{F} \log m_{\text{HCl}} + \frac{4.6RT}{F} \log \gamma_{\text{HCl}} \quad (13)$$

In moderately dilute solutions (see equation 13, page 82)

$$\log \gamma = -0.5\sqrt{m} + Bm \quad (14)$$

Combining 13 and 14 and rearranging, we have

$$E_0 - \frac{4.6RTB}{F} m_{\text{HCl}} = E - \frac{2.3RT}{F} \sqrt{m_{\text{HCl}}} + \frac{4.6RT}{F} \log m_{\text{HCl}} \quad (15)$$

The right side of equation 15 is plotted against  $m$  and the curve extrapolated to zero value of  $m$ . The intercept is equal to  $E_0$ .

<sup>7</sup> H. S. Harned and R. W. Ehlers, *J. Am. Chem. Soc.* 54, 1350 (1932).

<sup>8</sup> D. I. Hitchcock, *J. Am. Chem. Soc.* 50, 2076 (1928).



There are thus two independent methods, conductance measurements and electromotive force methods, for the evaluation of the true dissociation constant of a weak acid. Both methods involve the principle that at infinite dilution the activity coefficient of all substances is unity and at this concentration the stoichiometric concentrations are equal to the activities, i.e.,  $K_A$  equals  $K_c$ . The two methods agree quite well. Table 2 shows some thermodynamic dissociation constants determined by these methods.

TABLE 2

Thermodynamic Dissociation Constants of Some Weak Acids

Acid	25° C.	40° C.
Formic	$1.772 \times 10^{-4}$	$1.716 \times 10^{-4}$
Acetic	$1.754 \times 10^{-5}$	$1.705 \times 10^{-5}$
Chloroacetic	$1.378 \times 10^{-3}$	$1.229 \times 10^{-3}$
Propionic	$1.339 \times 10^{-5}$	$1.284 \times 10^{-5}$
n-Butyric	$1.515 \times 10^{-5}$	$1.395 \times 10^{-5}$
Benzoic		
$K_1$	$7.537 \times 10^{-3}$	$7.152 \times 10^{-3}$
$K_2$	$6.226 \times 10^{-8}$	$6.349 \times 10^{-8}$
Bisulfate ion	$12.0 \times 10^{-3}$	$9.73 \times 10^{-3}$
Hydrocyanic	$5.80 \times 10^{-10}$	$6.35 \times 10^{-10}$
Lactic	$1.37 \times 10^{-4}$	$1.367 \times 10^{-4}$

McTear and Belcher<sup>11</sup> have determined the first and second ionization constants of carbonic acid at 25° C. and at 38° C. using the glass electrode in place of the customary hydrogen electrode. They found  $K_1$  to be  $4.52 \times 10^{-7}$  at 25° C. and  $4.91 \times 10^{-7}$  at 38° C., while  $K_2$  was  $5.80 \times 10^{-11}$  at 25° C. and  $6.25 \times 10^{-11}$  at 38° C. Actually, the first dissociation constant is an apparent acid dissociation constant. This is because  $\text{CO}_2$  is only partially hydrated in water and we have the reaction



The true ionization constant of carbonic acid is evidently

$$K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \quad (16)$$

and the apparent first dissociation is

$$K_1' = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2] + [\text{H}_2\text{CO}_3]} \quad (17)$$

<sup>11</sup> M. T. McTear and J. W. Belcher, *J. Electrochem. Soc.*, **82**, 102 (1935); *Principles of Electrochemistry*, Reinhold Publishing Corp., New York, 1935, p. 102.

<sup>12</sup> J. H. Hildebrand and R. M. Joske, *J. Am. Chem. Soc.*, **55**, 2630 (1933); **57**, 1683 (1935).

The relation between these constants is

$$K_1 = K_1' + K_1' \frac{\text{CO}_2}{\text{H}_2\text{CO}_3} \quad (18)$$

It has been shown that at equilibrium the amount of dissolved unhydrated  $\text{CO}_2$  is about 1000 times the amount of hydrated  $\text{CO}_2$ , i.e., carbonic acid and, accordingly, the true dissociation constant of carbonic acid is about  $4.5 \times 10^{-4}$  which means that carbonic acid is actually about as strong an acid as formic acid.

The amount of  $\text{CO}_2$  that dissolves in water is proportional to the partial pressure of the gas over the solution<sup>11</sup> and

$$\text{Total CO}_2 = K_0 P_{\text{CO}_2} \quad (19)$$

where  $K_0$  is the solubility constant of  $\text{CO}_2$ .

The hydration of  $\text{CO}_2$  is a comparatively slow reaction, and it is only in the presence of the enzyme carbonic anhydrase that the reaction reaches equilibrium rapidly. Red blood cells are rich in carbonic anhydrase.<sup>12</sup>

Considerable work has been published on the influence of substituted groups on the ionization constants of acids. It is generally observed that the substitution of a strongly polar group greatly increases the strength of a carboxyl group. The closer the substituted polar group is to the carboxyl, the greater the influence on the dissociation of the carboxyl. For example, substituting a chlorine for a hydrogen atom in aliphatic acids gives the following  $pK_a$  values ( $pK_a$  is equal to  $\log 1/K_a$ ):

Position of chlorine	$\alpha$	$\beta$	$\gamma$	$\delta$
$pK_a$	2.86	4.10	4.52	4.70

A special example of the effect of substitution is presented by the dicarboxylic acids. It can be shown from statistical considerations that the first dissociation constant must be at least four times greater than the second. As a matter of fact, measurements have shown the first constant to exceed this limit considerably. Table 3 gives the ionization constants of some dicarboxylic acids.<sup>13</sup>

The influence of substituted groups on the ionization of organic acids has been discussed by Kirkwood and Westheimer.<sup>14</sup>

<sup>11</sup> D. D. Van Slyke, J. Sendroy, Jr., A. B. Hastings, and J. M. Neill, *J. Biol. Chem.* 78, 765 (1928).

<sup>12</sup> N. V. Meldrum and F. J. Roughton, *J. Physiol.* 75, 15 P (1932).

<sup>13</sup> R. Gane and C. K. Ingold, *J. Chem. Soc.* 1931, 2153.

<sup>14</sup> J. G. Kirkwood and F. H. Wertheimer, *J. Chem. Phys.* 6, 506 (1938).

TABLE 3

IONIZATION CONSTANTS OF SOME DICARBOXYLIC ACIDS

Acid	Formula	$K_1 \times 10^5$	$K_2 \times 10^6$	$K_1/K_2$
Malonic	$\text{HOOC}(\text{CH}_2)_1\text{COOH}$	149.0	2.0	734
Succinic	$\text{HOOC}(\text{CH}_2)_2\text{COOH}$	6.4	3.3	19.2
Glutaric	$\text{HOOC}(\text{CH}_2)_3\text{COOH}$	4.5	3.8	11.9
Adipic	$\text{HOOC}(\text{CH}_2)_4\text{COOH}$	3.8	3.9	9.8
Pimelic	$\text{HOOC}(\text{CH}_2)_5\text{COOH}$	3.2	3.7	8.7
Suberic	$\text{HOOC}(\text{CH}_2)_6\text{COOH}$	3.0	3.9	7.7
Azelaic	$\text{HOOC}(\text{CH}_2)_7\text{COOH}$	2.8	3.8	7.3

## THE pH SCALE

In living tissue, we deal with extremely dilute acids and bases. For example, the hydrogen-ion concentration of the human body is about  $0.000000105\text{ N}$ . Such a number is awkward to handle; more important still is the difficulty of comparing this value with stronger or weaker acids. Sometimes such a figure is expressed in powers of 10, that is, we write  $5 \times 10^{-7}$  instead of  $0.0000005$ . This method is still not entirely suited to our needs. The term pH was suggested by Sørensen<sup>15</sup> to help us out of this difficulty. Sørensen defined pH as

$$\text{pH} = -\log C_{\text{H}^+} = \log \frac{1}{C_{\text{H}^+}} \quad (20)$$

and likewise

$$\text{pOH} = -\log C_{\text{OH}^-} = \log \frac{1}{C_{\text{OH}^-}} \quad (21)$$

where  $C_{\text{H}^+}$  denotes the hydrogen-ion concentration and  $C_{\text{OH}^-}$  the concentration of hydroxyl ions.

Sørensen assumed that the concentration of hydrogen ions could be calculated from conductance measurements using Arrhenius' theory of dissociation. In this way he concluded that the hydrogen-ion concentration of a  $0.1\text{ N}$  HCl solution was  $0.09165$ , which corresponds to a pH of  $1.038$ . Then the hydrogen-ion concentration of a  $0.01\text{ N}$  HCl solution was calculated to be  $0.009165$  or a pH of  $2.038$ . We now know that this is not correct.

It is not possible to calculate accurately the hydrogen-ion concentration from conductance measurements with the methods used by Sørensen.

The pH scale is not a simple one. The correct formulation of pH is a complicated problem. The pH scale is defined as the standard against which all hydrogen electrodes are compared. It becomes imperative for the purpose of comparison that all electrodes are compared.

<sup>15</sup> *Journal of Biological Chemistry*, **8**, 1 (1909).



poses of thermodynamic calculations to formulate and calibrate the pH scale as accurately as possible.

Since the hydrogen electrode measures the hydrogen-ion activity rather than the hydrogen-ion concentration, a more useful definition of pH than that proposed by Sørensen is

$$pH = \log \frac{1}{A_{H^+}} \quad (22)$$

and likewise

$$pOH = \log \frac{1}{A_{OH^-}} \quad (23)$$

The meaning and nature of the pH scale is rather obvious, but perhaps it is well to point out two things about the scale. (1) It is not arithmetical: i.e., when a solution is brought from pH 7 to pH 6 the arithmetical increase in hydrogen-ion activity is only one-tenth of the increase in going from pH 6 to pH 5. (2) The scale is in inverse sense to the hydrogen-ion activity: i.e., the lower the pH, the higher is the hydrogen-ion activity.

As we have seen from Table 1, at 25° C. the ion activity product of water is very nearly  $1 \times 10^{-14}$ . Taking the logarithm of both sides of the expression for the ion activity product constant (equation 2) and multiplying by  $-1$ , we have very nearly

$$pH + pOH = pK_w = 14 \quad (24)$$

At pH 7 the pOH is also 7, and the reaction of water is acidic or basic to the same extent. Although absolutely pure water should have a pH of 7, the pH of the most carefully prepared water will seldom be exactly 7, owing to slight contamination.

The hydrogen ion is hydrated in solution; naked protons do not exist in water as such. The hydrated hydrogen ion is written  $H_3O^+$  and is called the hydronium ion. This fact, however, does not change any of our considerations, and we shall continue to speak of the hydrogen ion although it is understood that we mean the hydronium ion.

## CALIBRATION OF THE pH SCALE

The expressions for pH and pOH have been defined in terms of the activities of the hydrogen and hydroxyl ions, respectively. It now remains to calibrate the pH scale. That is to say, we must have some solution whose exact hydrogen-ion activity is known. This turns out to be a most troublesome problem for which there is no completely unambiguous answer.

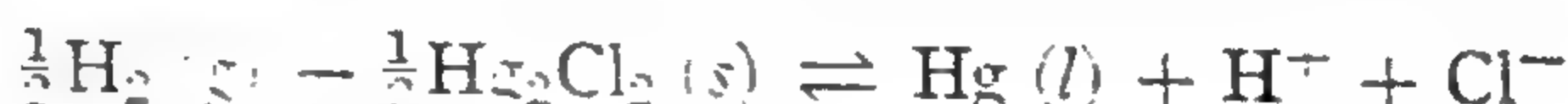
The hydrogen electrode is the standard that must be used to establish a pH scale. When the pH unit was first defined, it was believed that the

partial molar free energy of transfer of hydrogen ions,  $\Delta F$ , could be expressed exactly by the equation

$$\Delta F = RT \ln \frac{C_{H_1}}{C_{H_2}} = -FE \quad (25)$$

where  $C_{H_1}$  and  $C_{H_2}$  are the two concentrations of the hydrogen ion, and  $E$  is the difference in electromotive force of two concentration cells involving the hydrogen electrode and the calomel electrode.

The chemical reaction involved in the cell including the hydrogen and the calomel half cells is



and the emf of such a cell is

$$E = E_0 - \frac{RT}{F} \ln A_{H^+} A_{Cl^-} \quad (26)$$

It is evident that no matter what electromotive force cell we might employ we would always have to deal with a simultaneous transfer of an anion along with a hydrogen ion and, accordingly, what we actually measure is a mean ion activity which is  $\sqrt{A^+ \times A^-}$ . The additional complication of the liquid junction potential enters in. In principle, it is possible to construct a cell without a liquid junction for the measurement of hydrogen ions. However, in practice the solutions with which a biochemist has to deal often precludes the use of such a cell and a salt bridge of some kind must be used. The type of cell usually employed is



where the symbol  $||$  indicates a liquid junction. The equation for this cell in terms of pH may then be written

$$pH = \frac{F \cdot E - E_0'}{RT} \quad (27)$$

where  $E_0'$  is the voltage of the calomel half cell and includes the liquid junction potential as well as the fact that the anion activity may not be the same as the hydrogen ion activity. The problem is then to evaluate the magnitude of  $E_0'$ . Cohn and his co-workers<sup>16</sup> were apparently the first to attempt this evaluation by the use of weak acids in the presence of their salts and by making use of the ionization constant of the weak acid in their calculation.

<sup>16</sup> J. C. Cohn, *J. Biol. Chem.*, **50**, 173 (1927).

<sup>17</sup> J. C. Cohn, L. E. Heydich, and M. E. Menkin, *J. Am. Chem. Soc.*, **50**, 696 (1928).

We have already described the method by which the thermodynamic ionization constant of a weak acid can be measured. The proposal was made that a weak acid in the presence of its salt of a strong base be used in the solution in contact with the hydrogen electrode. The pH of such a solution can be approximately calculated by the relation

$$\text{pH} = \text{p}K_A + \log \frac{\text{Salt}}{\text{Acid}} \quad (28)$$

The ratio of salt to acid is fixed at unity but the total concentration varied. The pH of the solutions is calculated using selected values for  $E_0'$  and these calculated values of pH are extrapolated back to zero concentration. The intercept at zero concentration should equal  $\text{p}K_A$  which was measured as described previously in cells without liquid junction potentials. The value of  $E_0'$  which yields the correct extrapolated value of  $\text{p}K_A$  is selected as the normal potential of the cell. The pH is thus substantially defined in terms of the potential of the half cell employed. Hitchcock and Taylor<sup>17</sup> applied the Cohn method to a number of systems using the correct thermodynamic dissociation constants. MacInnes, Belcher, and Shedlowsky<sup>18</sup> extended this type of measurement.

It turns out that this more modern technique for the calibration of the pH scale is about 0.04 pH units higher than the Sørensen scale.<sup>19</sup>

Whereas the pH scale has been calibrated as well as the present theory permits, the question of the single-ion activities remains unanswered, and, accordingly, the scale does not represent a standard in the accepted sense of this term.

Table 4 shows the pH of some standard buffer solutions at 25° C. which have been evaluated by the techniques described above.

TABLE 4  
pH OF STANDARD BUFFER SOLUTIONS AT 25° C.

Solution	pH
Acid potassium phthalate (0.05 M)	4.01
Acetic acid (0.01 M) + sodium acetate (0.01 M)	4.71
Acetic acid (0.1 M) + sodium acetate (0.1 M)	4.65
Potassium dihydrogen phosphate (0.01 M) + disodium hydrogen phosphate (0.01 M)	6.97
Potassium dihydrogen phosphate (0.025 M) + disodium hydrogen phosphate (0.025 M)	6.87
Borax (0.01 M)	9.18

<sup>17</sup> D. I. Hitchcock and A. C. Taylor, *J. Am. Chem. Soc.* 59, 1812 (1937).

<sup>18</sup> D. A. MacInnes, D. Belcher, and T. Shedlowsky, *J. Am. Chem. Soc.* 60, 1094 (1938).

<sup>19</sup> R. G. Bates, *Chem. Revs.* 42, 1 (1948).



## DETERMINATION OF HYDROGEN IONS

It is not necessary to elaborate extensively on this topic; methods of measurement are adequately treated in several excellent textbooks.<sup>20</sup>

Two general methods are available for the determination of hydrogen ions: (1) the indicator method, and (2) the electrometric method. The indicator method depends upon the fact that various organic substances have a particular color in an acid solution. The shade or tint of the color, at least over a range of about 2  $pH$  units, is a function of the  $pH$  of the solution. Accordingly, a series of buffers of known  $pH$  is made up and a definite amount of the selected indicator added to each. The same amount of indicator is added to the unknown solution, and the color of the unknown is compared with the colors of the buffer series. The closest matching color is found and the  $pH$  estimated directly. This method is useful for routine work where conditions can be rigorously controlled. Indicators are subject to a number of errors, however, and, in general, the electrometric method is to be greatly preferred.

The electrometric method depends on the measurement of the electromotive force of a cell whose potential is a function of  $pH$ . The hydrogen electrode is the classical electrode for this purpose. It consists of a piece of inert metal such as gold or platinum covered with platinum black by electrolyzing a solution of platinic chloride. The platinum black is saturated with hydrogen gas and maintained in this condition. The hydrogen is adsorbed on the surface of the platinum black, and for our purposes the electrode becomes a strip of hydrogen. The hydrogen electrode is put into the solution under investigation and connected with a half cell. This arrangement may or may not involve a liquid junction. If a calomel half cell is used, as is the common practice, it is connected with the solution under investigation with a potassium chloride salt bridge. This, of course, involves a liquid junction. The voltage of the hydrogen electrode—calomel electrode is measured with an accurate potentiometer. The  $pH$  is directly proportional to the electromotive force, and the  $pH$  is calculated by the formula

$$pH = \frac{E - E_0}{\frac{RT}{F}} \quad (29)$$

where  $E$  is the voltage measured with the potentiometer and  $E_0$  is the contribution made by the calomel half cell. We have seen in a previous sec-

<sup>20</sup> W. M. Clark, *Determination of Hydrogen Ions*, Williams and Wilkins Co., Baltimore, 1928.

L. Michaelis, *Hydrogen Ion Concentration*, Williams and Wilkins Co., Baltimore, 1926.

I. M. Kolthoff and H. A. Laitinen, *pH and Electro Titrations*, John Wiley & Sons, Inc., New York, 1941.

tion how  $E_0$  is evaluated by means of the ionization constant of a weak acid. Other electrodes than the hydrogen electrode can be used to measure hydrogen-ion activity.

The quinhydrone electrode enjoyed wide popular favor before the advent of the glass electrode. Quinhydrone consists of equimolecular mixture of hydroquinone (reductant) and of quinone (oxidant). It is dissolved in the unknown solution, and an inert metal electrode such as platinum is inserted into the solution and connected through a salt bridge to a calomel half-cell. An oxidation-reduction potential is realized which is proportional to the  $pH$ ; we shall see in Chapter 8 why such an oxidation-reduction electrode is responsive to hydrogen ions. The quinhydrone electrode cannot be used above  $pH$  8.0, and it also fails in the presence of oxidizing and reducing substances.

Owing to ease of operation, the glass electrode has almost entirely superseded all other methods for the evaluation of  $pH$ . Cremer discovered the glass electrode, but the electrode was developed into a practical instrument by Haber and Klemensiewicz.<sup>21</sup> There are many commercial instruments of excellent design, and Dole<sup>22</sup> has written an outstanding treatise on the subject.

The glass electrode consists of a glass membrane of suitable composition on one side of which is a non-polarizable electrode, such as silver-silver chloride. On the other side is the unknown solution, which is connected by means of a salt bridge to another non-polarizable electrode. The arrangement is shown in Fig. 2.

The observed voltage is a straight line function of  $pH$ ; the slope of this relation, however, varies with temperature. At 25° C. the voltage changes 59 millivolts per unit  $pH$ ; whereas the slope of the  $pH$ -emf line is dependent only on temperature, the position of the line has to be fixed by the use of a buffer of known  $pH$ , i.e., the glass electrode has to be calibrated. The observed voltage must be measured with a quadrant electrometer or as usually done with a vacuum-tube potentiometer because the electrical resistance of the glass membrane is too high to permit the use of a measuring device that draws an appreciable current.

The advantages of the glass electrode are: (1) the glass electrode is independent of oxidation-reduction potentials, (2) it is not necessary to pass a gas through the solution or to add any material to it, (3) it is possible to use very small quantities of solution, (4) the electrode can be used in colored or turbid solutions, (5) the electrode gives accurate values in poorly buffered solutions, (6) equilibrium is rapidly reached.

<sup>21</sup> M. Cremer, *Biochem. Z.* 47, 562 (1906).

F. Haber and Z. Klemensiewicz, *Z. physik. Chem.* 67, 385 (1909).

<sup>22</sup> M. Dole, *The Glass Electrode*, John Wiley & Sons, New York, 1941.

It should be remembered, however, that the glass electrode is subject to errors both in the extreme acid as well as in the extreme alkaline region. Above a  $pH$  of about nine, the  $pH$  as measured by the glass electrode is less than the true  $pH$  and this effect becomes progressively more pronounced as the  $pH$  is increased. Qualitatively, it is easy to see why such errors might be found on the alkaline side. In this region the concentration of cations other than hydrogen ions is about  $10^9$  greater than that of the

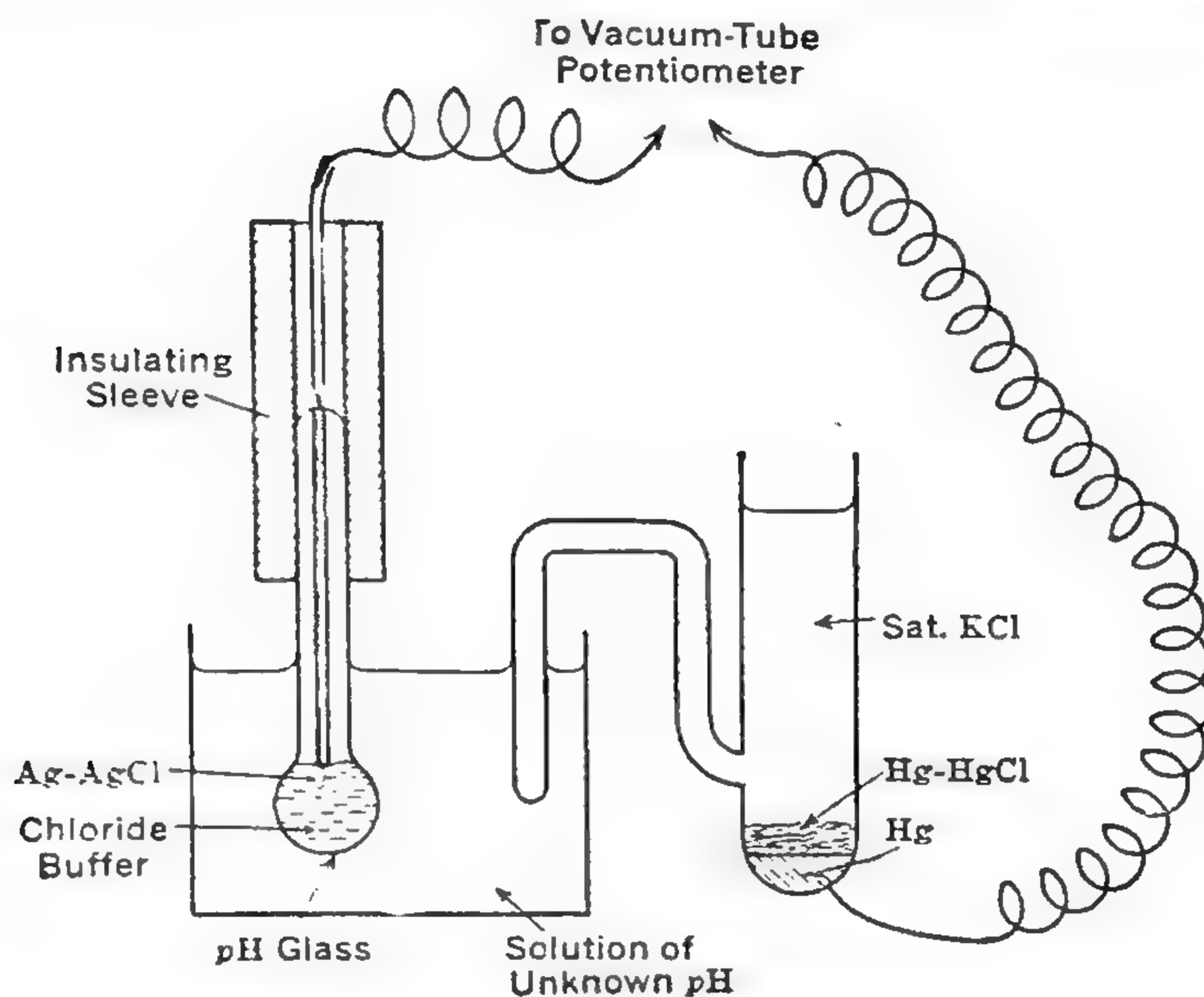


FIG. 2. Glass electrode for the measurement of  $pH$ .

hydrogen ions. This enormous excess of cations over the hydrogen ions would be expected to influence the potential. The different glasses vary considerably in the magnitude of their alkaline error.

On the acid side, below  $pH$  0, we again find deviations from the straight-line relation between  $pH$  and the electromotive force. In this region the errors are negative, i.e., the calculated  $pH$  is greater than the actual  $pH$ . In order to understand the deviation in the extreme acid region, we must have a look at the theory of the glass electrode.

The potential developed across the glass membrane is believed to be due to the fact that, under ordinary circumstances, only hydrogen ions can enter the surface of the glass. In short, the potential can be treated as a diffusion potential, the magnitude of which, as we have seen in the previous chapter, is given by

$$E = \frac{v - u}{v + u} \frac{RT}{F} \ln \frac{A_2}{A_1} \quad (30)$$



$v$  is the mobility of the anion; if the glass is impermeable to anions,  $v$  is zero and

$$E = - \frac{RT}{F} \ln \frac{A_2}{A_1} \quad (31)$$

Accordingly, the electromotive force is dependent on the difference in the hydrogen-ion activity as indicated. As explained previously, we do not have naked protons ( $H^+$ ) in solution, but the hydrogen ions are hydrated to form  $H_3O^+$  and this is the form in which the protons enter the glass membrane. The glass electrode thus acts as a water electrode since it is concerned with the transport of the hydrated proton. In the extreme acid region, the activity of the water is decreased by the high acid concentration and this decreased water activity leads to a decrease in the electromotive force of the electrode. On the other hand, Sinclair and Martell<sup>23</sup> claim that the acid errors of the glass electrode are too great to be due to decreased water activity alone and suggest that there is an anion error.

Haugaard<sup>24</sup> has studied the conductance across glass-electrode membranes. He believes that the glass electrode performs as a hydrogen electrode, owing to the ability of glass to exchange sodium ions for hydrogen ions. His picture is somewhat as follows: The glass takes up water, and the sodium salts of silicic acid dissociate. The hydrogen ions displace the sodium ions from the surface of the glass, forming a skeleton of silicic acid. There remains, however, in the center of the glass membrane a layer of intact sodium salt. With the passage of current through the membrane during the compensation of the electromotive force of the system, there is a slight movement of the salt layer towards one side or the other, depending on the direction of the current. The hydrogen ions, however, are the only ions that can enter the surface of the glass, and, accordingly, the glass-electrode may be looked upon as a membrane that is permeable only to hydrogen ions.

#### APPROXIMATE CALCULATION OF $pH$

The occasion frequently arises in experimental work when it is desirable to calculate the approximate value of the  $pH$  of a solution. For pure solutions of strong acids such as hydrochloric, or solutions of these acids in the presence of salts of strong acids and strong bases such as  $KCl$  or  $NaCl$ , a fairly close value can be obtained simply by taking the logarithm of the reciprocal of the acid concentration. Thus, the  $pH$  of a 0.001  $N$   $HCl$  solution is very nearly 3.

<sup>23</sup> E. E. Sinclair and A. E. Martell, *J. Chem. Phys.* 18, 224 (1950).

<sup>24</sup> G. Haugaard, *J. Phys. Chem.* 45, 148 (1941).

The calculation of the  $pH$  of a pure solution of a weak acid can be made, provided that we know the ionization constant of the acid. In such a case the hydrogen-ion concentration is equal to the anion concentration, and we can write

$$\frac{(H^+)^2}{\text{Acid}} = K_a \quad (32)$$

or

$$H^+ = \sqrt{K_a \times \text{Acid}} \quad (33)$$

Taking the logarithms of both sides of equation 33 and rearranging, we obtain

$$pH = \frac{1}{2}pK_a - \frac{1}{2}\log \text{Acid} \quad (34)$$

where the acid concentration is really that of the undissociated acid, but for most purposes can be taken equal to the total acid concentration.

During the titration of a weak acid such as acetic acid by a strong base such as sodium hydroxide, the equation

$$\frac{H^+ \times A^-}{HA} = K_a \quad (35)$$

holds throughout the titration. Since, however, the acid is ionized to a very limited extent, practically all the anions come from the ionization of the salt formed by titration, so that we may, without making any considerable error, write

$$\frac{H^+ \times \text{Salt}}{\text{Acid}} = K_a \quad (36)$$

Taking logarithms of both sides of the equation and rearranging, we obtain

$$pH = pK_a + \log \frac{\text{Salt}}{\text{Acid}} \quad (37)$$

where "acid" is taken as the total amount of acid present, i.e., both dissociated and undissociated. Equation 37 is very useful and important. The  $pH$  of a buffer can be calculated with it. Note that, if the acid is just neutralized, the  $pH$  equals the  $pK_a$ . Also note that the  $pH$  is independent of dilution and depends only on the ratio of salt to acid; although this is not entirely true, it is true enough for a great many purposes. A corresponding analogous equation can be derived for the titration of a weak base with a strong acid.

It is a common experience that salts of strong acids and strong bases are neutral reactions. For example,  $\text{NaCl}$ ,  $\text{K}_2\text{SO}_4$ , etc., dissolved in water are neutral. On the other hand, a solution of sodium acetate is alkaline and ammonium chloride is acid.

Sodium acetate is practically completely ionized in solution



The acetate ion so formed reacts with water



The acetate ion acts in this instance as a base in the Brönsted sense, since it is able to accept a proton from water.

For the acetic acid formed from the hydrolysis of sodium acetate, we can write the usual equilibrium equation

$$\frac{[\text{H}^+] \times [\text{Ac}^-]}{[\text{HAc}]} = K_a \quad (38)$$

and rearranging

$$[\text{H}^+] = \frac{K_a [\text{HAc}]}{[\text{Ac}^-]} \quad (39)$$

The amount of acetic acid formed equals the amount of hydroxyl ions produced, and the amount of acetate ion very nearly equals the amount of sodium acetate (salt) in solution. When this information is incorporated in equation 39, there results

$$[\text{H}^+] = \frac{K_a \times [\text{OH}^-]}{[\text{Salt}]} \quad (40)$$

Since

$$[\text{OH}^-] = \frac{K_w}{[\text{H}^+]} \quad (41)$$

we have from 40 and 41

$$([\text{H}^+])^2 = \frac{K_a K_w}{[\text{Salt}]} \quad (42)$$

Taking logarithms of both sides of equation 42 and multiplying through by a minus one, we have

$$pH = 7 + \frac{1}{2}pK_a + \frac{1}{2} \log [\text{Salt}] \quad (43)$$

Similarly, for a salt of a strong acid and a weak base, for example, ammonium chloride

$$pH = 7 - \frac{1}{2}pK_b - \frac{1}{2} \log [\text{Salt}] \quad (44)$$

where  $K_b$  is the ionization constant of ammonium hydroxide.

Employing the same line of reasoning, we find that the  $pH$  of a solution of a salt of a weak acid and a weak base, for example, ammonium acetate, is

$$pH = 7 + \frac{1}{2}pK_a - \frac{1}{2}pK_b \quad (45)$$

The  $pH$  of a solution of such a salt is independent of concentration.



## TITRATION CURVES

If acids are titrated with bases or bases with acids and the  $pH$  of the solution is determined as the titration is carried out, a titration curve can be drawn. One axis of such a graph is  $pH$  and the other the amount of acid or base added. Such titration curves have shapes characteristic of the acids and bases employed. Titration curves are shown in Fig. 3.

The subject of titration curves brings us directly to the next topic, buffers.

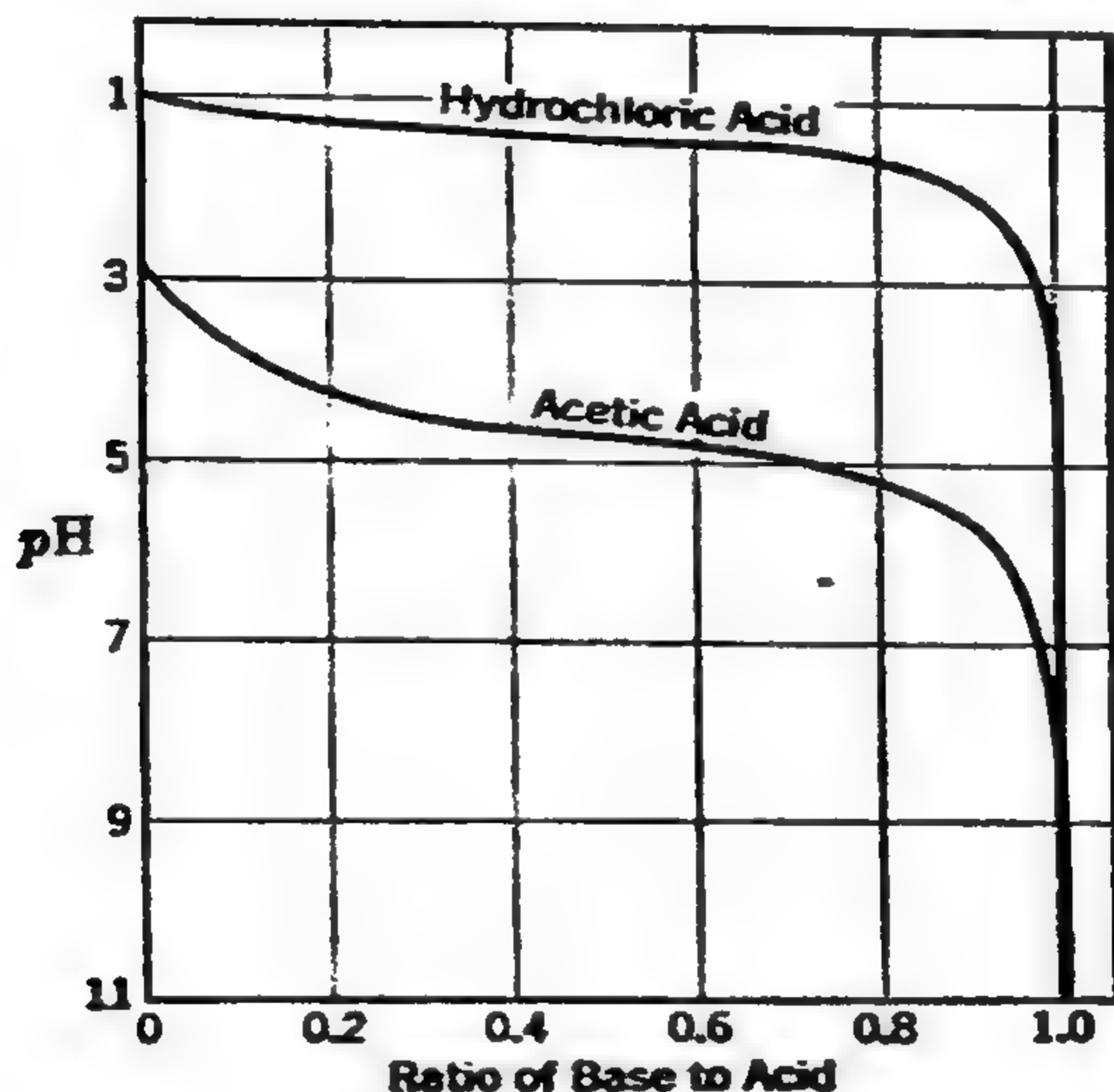


FIG. 3. Titration curves of 0.1 *N* acids and bases.

## BUFFERS

Buffers are defined as substances that resist changes in the  $pH$  of a system. All weak acids or bases in the presence of their salts form buffer systems. Examine the titration curve of acetic acid by sodium hydroxide shown in Fig. 3. It will be noted that the  $pH$  changes least for a given addition of base at the half neutralization point. The slope of the titration curve is at a minimum here, and the buffer capacity is at a maximum. The  $pH$  also equals the  $pK_a$ .

Buffer capacity is the differential quotient of the increasing amount of base expressed in equivalents per liter, over the corresponding change of  $pH$ . It is the reciprocal of the slope of the titration curve when the amount of acid or base is expressed in equivalents. Buffer capacity is usually denoted by the symbol  $\pi$ . In short,  $\pi$  equals  $\frac{dB}{dpH}$  or  $\frac{dA}{dpH}$ . Figure 4 shows

how the buffer capacities of several buffer systems vary with  $pH$  of the solution. Included in Fig. 4 is the buffer capacity of whole dog blood. Note that the maximum buffer capacity of blood covers the physiological range. This is as it should be.

Buffers are, in general, used for two purposes: (1) as a reference standard for  $pH$  determinations, and (2) to maintain the proper acid-base reaction of a medium such as a bacterial culture or an enzymatic reaction mixture.

The selection of the proper buffer systems for a given experimental use is a common problem. In the extreme acid region, hydrochloric acid of the proper concentration is usually chosen. In the intermediate region of  $pH$  3 to 4 phthalic acid-potassium acid phthalate can be used, and from  $pH$  4 to 6 acetic acid-sodium acetate serves well. The monosodium dihydrogen phosphate (acid)-disodium monohydrogen phosphate (salt) system

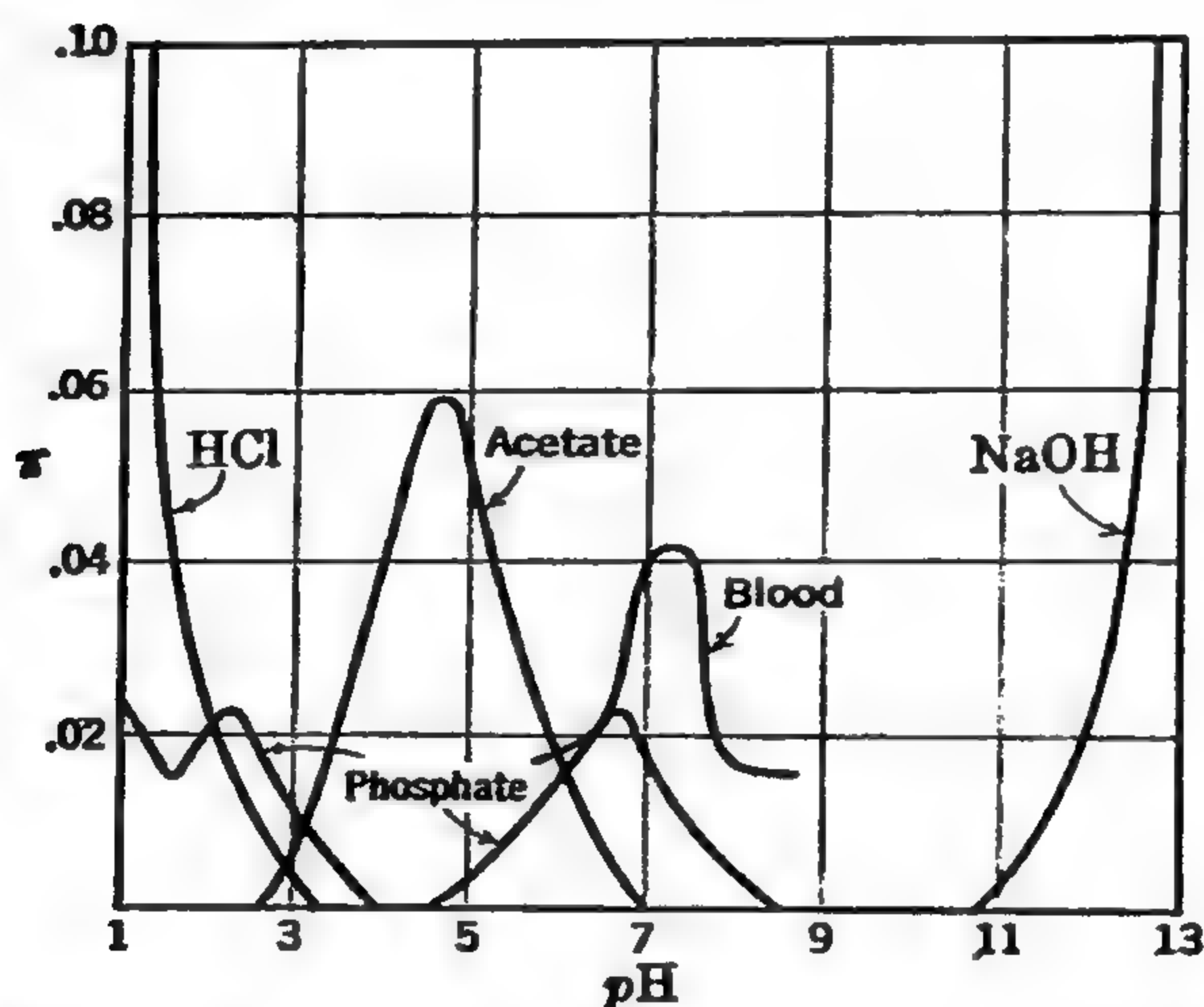


FIG. 4. Buffer capacities of several 0.1  $M$  buffer systems as a function of  $pH$ . Also included is the buffer capacity of whole dog blood.

covers the range from  $pH$  6 to 8. The more concentrated the buffer, the greater is its buffer capacity and the wider is the  $pH$  range it will cover.

The proper ratio of salt to acid to yield the desired  $pH$  can be calculated with the aid of equation 37. The relative success of such a calculation depends on how the apparent dissociation constant of the acid varies with concentration. The variation of the apparent dissociation constant of acetic acid with ionic strength is small, and, accordingly, it is usually possible to make up acetate buffers with  $pH$  values very close to those calculated. Such calculations involving the second hydrogen of phosphoric acid are not nearly so successful. Shown in Table 5 are values of the apparent  $pK_a$  of acetic and of the second hydrogen of phosphoric acid as a function of the ionic strength.

Sometimes the conditions of the experiment demand that the ionic strength of the buffer remain constant as the  $pH$  is varied over a certain range. In this event, the ionic strength is calculated by equation 3 of

TABLE 5

APPARENT  $pK_a$  VALUES OF ACETIC ACID AND THE SECOND HYDROGEN OF PHOSPHORIC ACID AT VARIOUS IONIC STRENGTHS AT 25° C.

Ionic Strength	$pK_a$ Values	
	Acetic	Phosphoric
0	4.730	7.16
0.316	4.725	7.02
0.448	4.722	6.93
0.632	4.717	6.80
0.706	4.716	6.75
0.836	4.717	6.68
0.894	4.718	6.64
1.00	4.721	6.60

Chapter 4 and the concentration of the buffers is adjusted until the ionic strength is constant. In making such a calculation it is assumed that all neutral salts are completely ionized. Disodium monohydrogen phosphate ionizes as follows:



and the ionic strength of a 0.1 *M* solution of this salt is

$$\mu = \frac{0.1 \times 1^2 + 0.1 \times 1^2 + 0.1 \times 2^2}{2} = 0.3$$

Biochemical experiments frequently cover a wide range of *pH* values and extend beyond the ranges of the buffer action of any one system. It then becomes necessary to use two or more of the systems to cover the desired range of *pH*. This introduces certain specific ion effects which may assume considerable physiological and biochemical importance, and the effect of the variation of hydrogen ions may be lost.

Michaelis<sup>25</sup> proposed a "universal" buffer which has an adequate buffer capacity over a very wide range of *pH* values. The stock solution of this buffer contains sodium acetate, the sodium salt of veronal, and sodium chloride. To this stock solution is added 0.1 *N* hydrochloric acid in the amounts indicated in the publication. The veronal system has the advantage that it forms no insoluble calcium salt. It covers the range from *pH* 2.5 to 9.5.

Teorell and Stenhagen have also prepared a so-called universal buffer which has been modified by Östling and Virtama<sup>26</sup> and covers the range

<sup>25</sup> L. Michaelis, *Biochem. Z.* 234, 139 (1931).

<sup>26</sup> S. Östling and P. Virtama, *Acta Physiol. Scand.* 11, 289 (1946).



from  $pH$  2 to 12. This buffer is made up in a stock solution containing disodium phosphate, citric acid, boric acid, and sodium hydroxide. To obtain the desired  $pH$ , 0.1  $N$  hydrochloric acid is added in the amounts indicated by the authors.

#### BUFFERS OF LIVING SYSTEMS

Living systems require the maintenance of  $pH$  within narrow limits, and it is therefore necessary for these systems to make extensive use of buffers. The most effective buffer system is protein which can take up and release relatively large quantities of hydrogen ions.

As the living cell respire, carbon dioxide is being continually produced, part of which is converted into bicarbonate and forms a buffer system. The  $pH$  of the bicarbonate system is given by the relation

$$pH = 6.11 + \log \frac{HCO_3^-}{CO_2} \quad (46)$$

where  $CO_2$  is the concentration of hydrated plus the unhydrated  $CO_2$  present in solution. The amount of  $CO_2$  so dissolved is a function of the partial pressure of carbon dioxide in the gas phase, and, accordingly, from a knowledge of the  $pH$  and the bicarbonate concentration, the partial pressure of  $CO_2$  in any tissue may be calculated.

The buffer system of mammalian blood and the transformation that these systems undergo are fairly complex. There is a release of carbon dioxide from the lungs with the uptake of oxygen by the hemoglobin in the red cells. The oxyhemoglobin is a significantly stronger acid than is reduced hemoglobin, and, accordingly, the uptake of oxygen tends to liberate carbon dioxide. As the blood passes through the capillaries, carbon dioxide is released to it from the tissues and at the same time oxyhemoglobin releases oxygen to the tissues. The blood then completes the cycle by returning to the lungs. The exchange of electrolytes between the red cells and the plasma and the mechanics of respiration are a complex but interesting problem.<sup>27</sup>

#### ZWITTER IONS

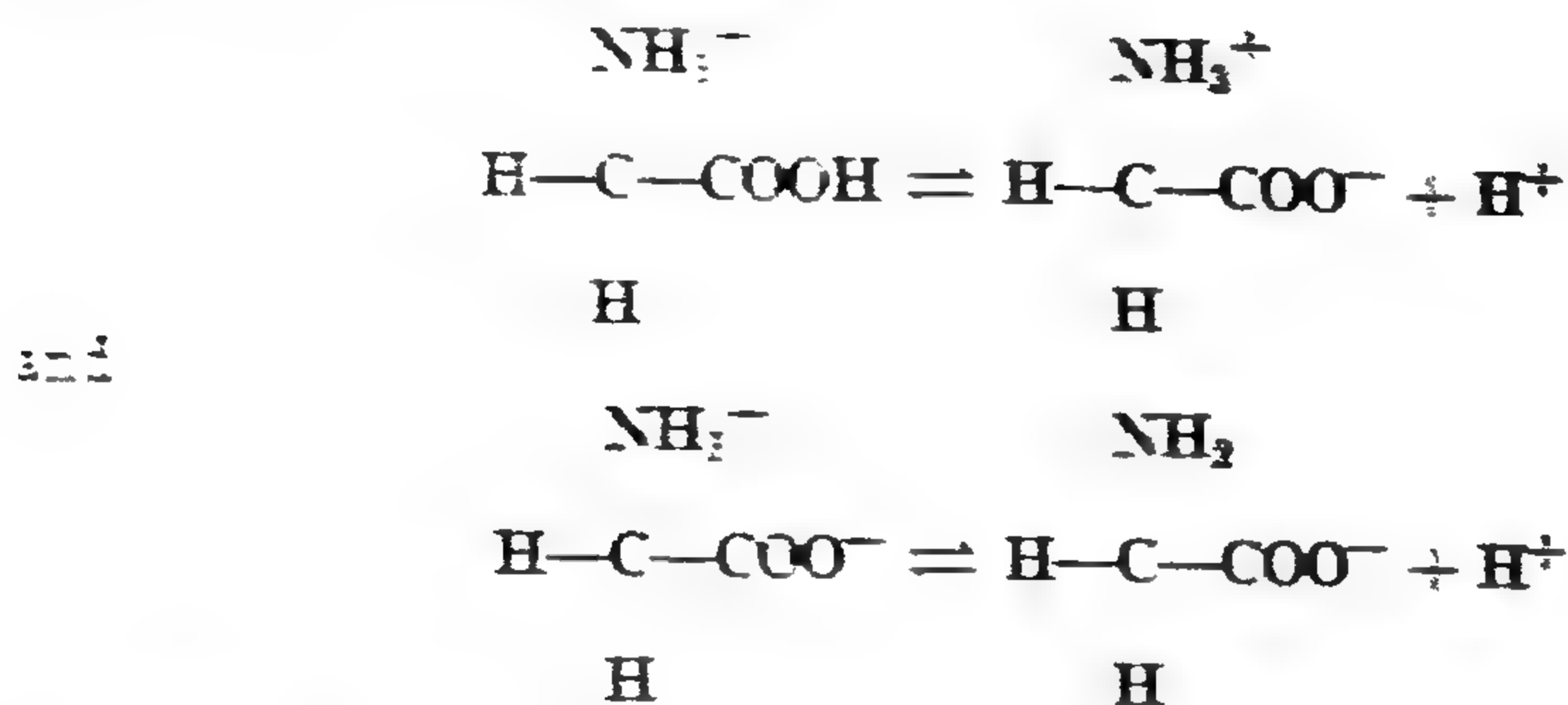
Amino acids, proteins, and phospholipids in the appropriate  $pH$  zone form what are known as zwitter ions (also called dipolar ions). The suggestion was first made by Adams<sup>28</sup> and later by Bjerrum<sup>29</sup> that an amino acid such

<sup>27</sup> W. M. Clark, *Topics in Physical Chemistry*, Williams and Wilkins, Baltimore, 1948.

<sup>28</sup> E. Q. Adams, *J. Am. Chem. Soc.* 38, 1503 (1916).

<sup>29</sup> N. Bjerrum, *Z. physik. Chem.* 104, 147 (1923).

as glycine ionizes as follows:



The acid dissociation constants for these two ionizations are

$$K_1 = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} \quad (47)$$

$$K_2 = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}^-]} \quad (48)$$

If a monoamino monocarboxylic acid such as glycine be titrated with acid and with base, a titration curve such as shown in Fig. 5 is obtained.

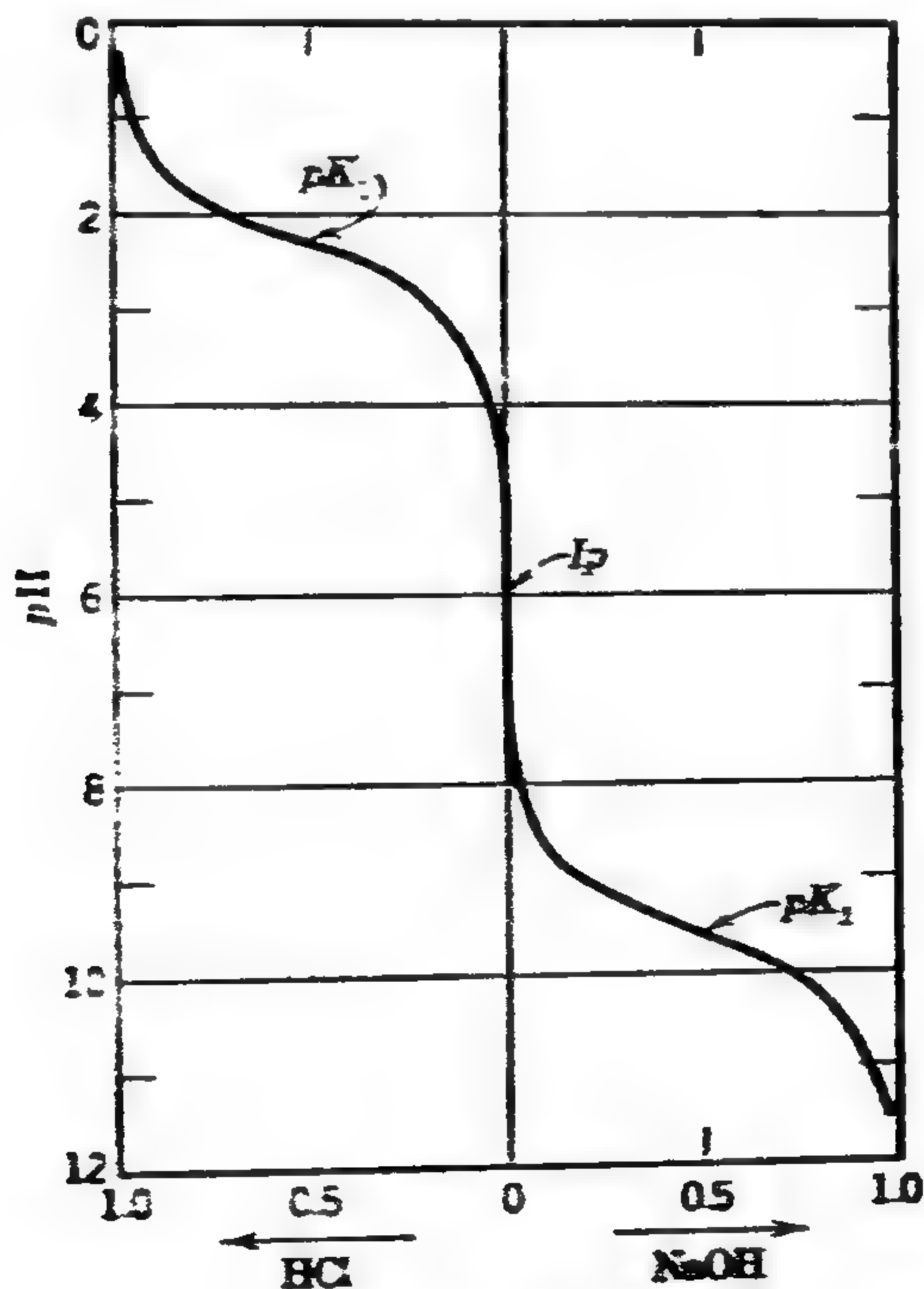


FIG. 5. Titration curve of glycine.

From what has been discussed, the mid-point of neutralization gives the  $pK_a$  of an acid. From inspection of Fig. 5 there are evidently two  $pK_a$  values for glycine corresponding to the two protons released by the cationic form of glycine. These values are  $pK_1$  equal to 2.34 and  $pK_2$  equal to 9.60.

For amino acids such as aspartic which has two carboxyl groups and one amino group and for histidine with one carboxyl, one imino, and one amino, the titration curves are naturally more complex than the curve for glycine.

### ISOELECTRIC AND ISOIONIC POINTS

The isoelectric point is defined as that  $pH$  at which the net positive and negative charge on a particle or on a surface is zero and there is no net motion in an electric field. The isoionic point, on the other hand, is defined<sup>30</sup> as that  $pH$  at which the number of positive and negative groups which arise exclusively from proton exchange are equal to each other. It is evident that the isoelectric and isoionic points are identical only if the zwitter ion combines with no ions other than hydrogen ions. The isoelectric point is properly measured with electrokinetic techniques and will be discussed in Chapter 9. The isoionic point of a protein may be determined by dialyzing a protein solution until all foreign ions are removed; the  $pH$  of the resulting protein will equal its isoionic point. Scatchard and Black<sup>31</sup> have discussed the effects of salts on the isoionic and isoelectric points of proteins.

The  $pH$  of the isoionic point of a simple zwitter ion such as an amino acid may be calculated as follows:<sup>32</sup> Combining equations 47 and 48 and eliminating the concentration of the zwitter ion, we have

$$H^2 = \frac{K_1 A^+ K_2}{A^-} \quad (49)$$

and, since by definition  $A^+$  is equal to  $A^-$ , there results from equation 49

$$H = \sqrt{K_1 K_2} \quad (50)$$

Taking logarithms of both sides of equation 50 and rearranging, we have

$$pH_i = \frac{1}{2}(pK_1 + pK_2) \quad (51)$$

Amino acids in solution exist over a broad range of  $pH$  as cations, zwitter ions, and anions. Thus if we set

$$A^+ + A^- + {}^+A^- = 1 \quad (52)$$

<sup>30</sup> S. P. L. Sørensen, K. Linderström-Lang, and E. Lund, *J. Gen. Physiol.* 8, 543 (1927).

<sup>31</sup> G. Scatchard and E. S. Black, *J. Phys. & Colloid Chem.* 53, 88 (1949).

<sup>32</sup> L. Michaelis, *Die Wasserstoffionenkonzentration*, Julius Springer, Berlin, 1914.



and substitute equations 47 and 48 and rearrange, we can calculate the fractions of the amino acid in solution which exist as cation, as zwitter ion, and as anion. Figure 6 shows the results of such calculations for glycine.

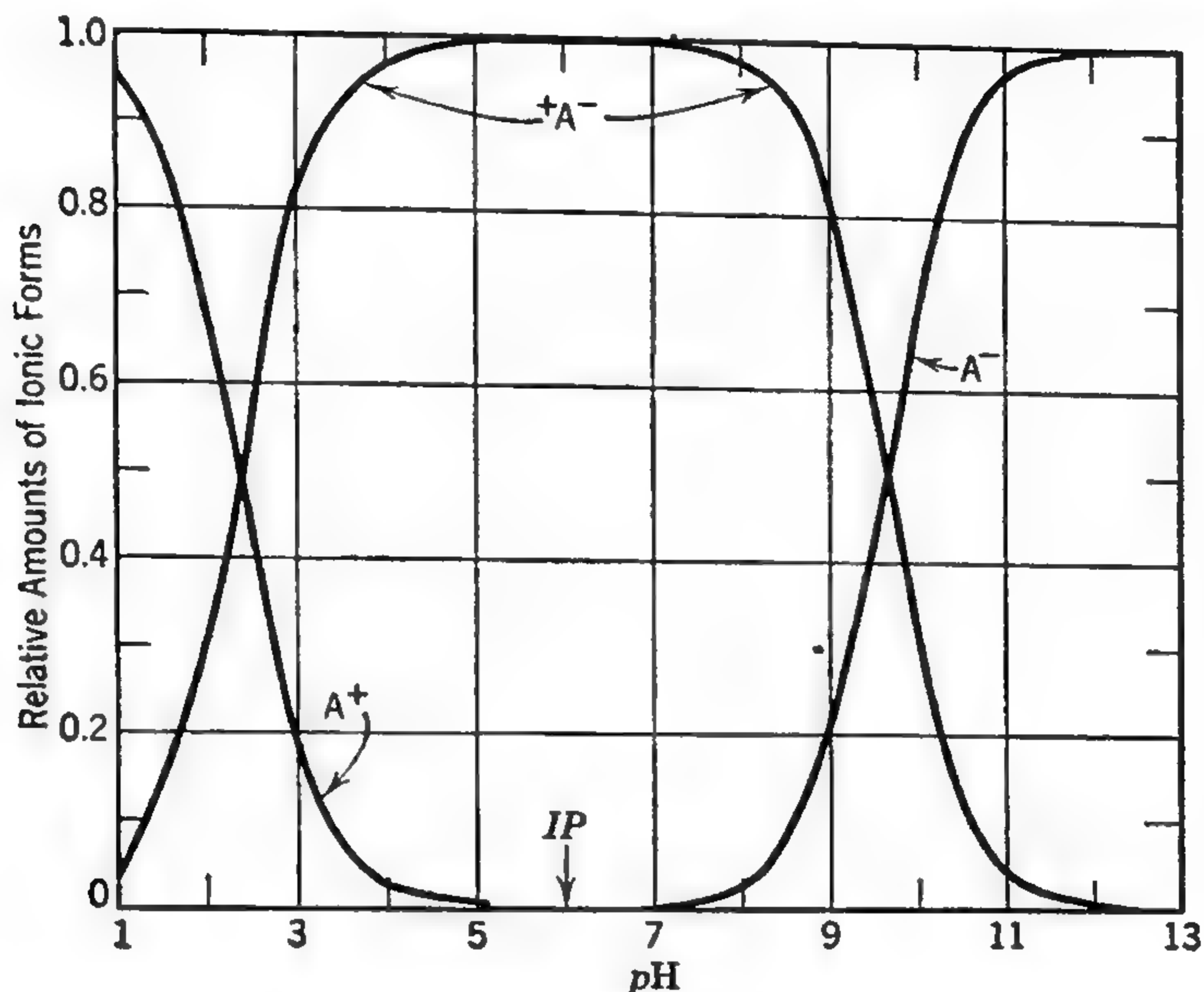


FIG. 6. Relative ionic composition of a glycine solution as a function of  $pH$ .

Shown in Table 6 are the dissociation constants and isoionic points of some amino acids at  $25^{\circ}C$ .

TABLE 6

$pK$  AND ISOIONIC VALUES FOR SOME AMINO ACIDS AT  $25^{\circ}C$ .

Acid	$pK_1$	$pK_2$	$pK_3$	$pH_i$
Glycine	2.34	9.60		5.97
Alanine	2.34	9.69		6.00
	2.36	9.60		6.04
Aspartic	1.88	3.65	9.60	2.77
Glutamic	2.19	4.25	9.67	3.22
Histidine	1.82	6.00	9.17	7.59
Lysine	2.18	8.95	10.53	9.74
Arginine	2.17	9.04	12.48	10.76

### ACID-BASE TITRATION OF PROTEINS

As is well known, proteins are made up of amino acids linked together through peptide bonds. A certain fraction of these amino acid residues have acidic or basic groups, and it is to these groups that proteins owe their acid-base properties. Proteins exist in their isoionic zone as zwitter ions in

the same sense as do amino acids. A segment of a hypothetical peptide chain containing exposed ionogenic groups is shown in Fig. 7.

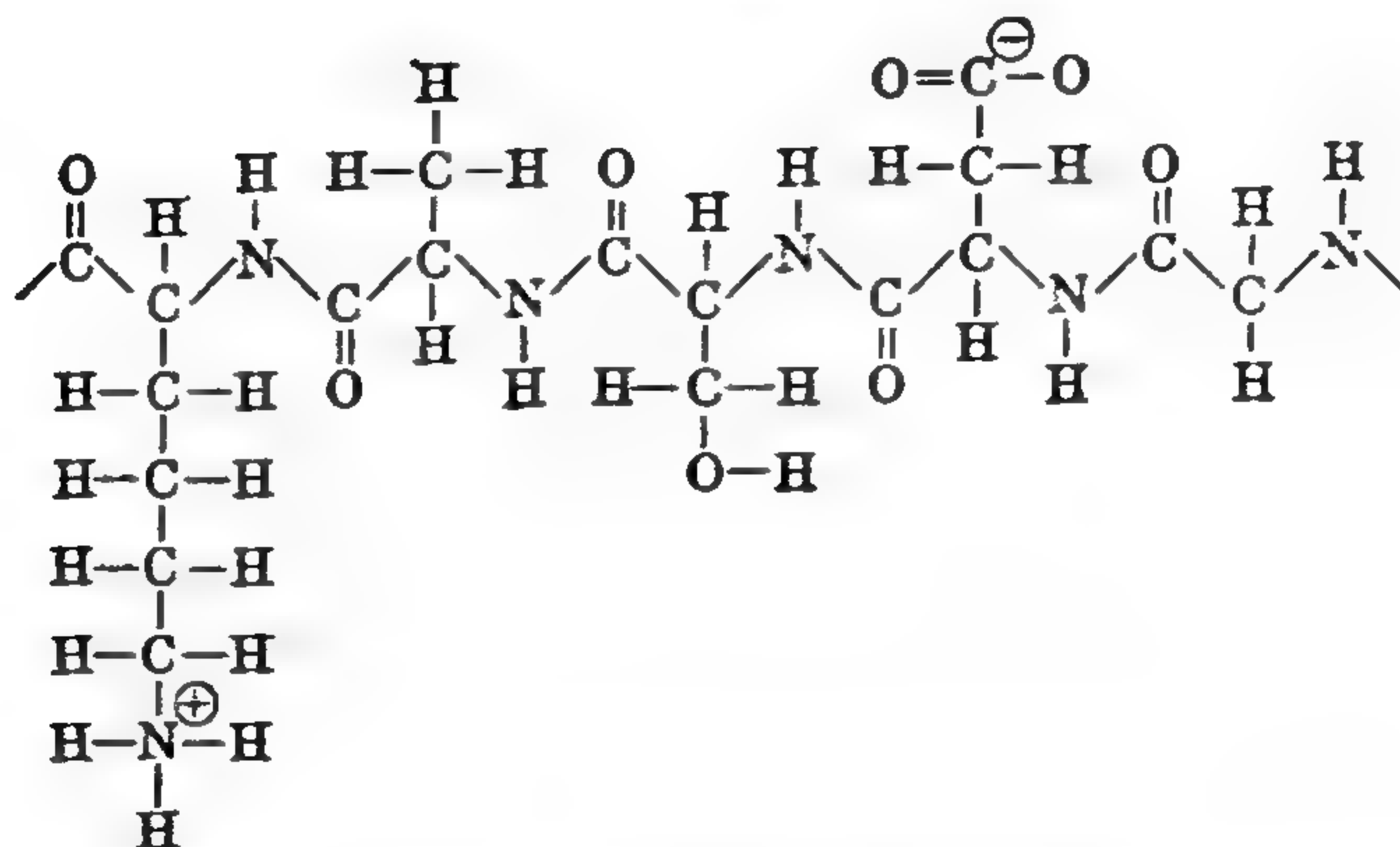


FIG. 7. Segment of hypothetical peptide chain.

Many examples of acid-base dissociation curves may be found in the literature.<sup>33</sup> The proteins that have been most studied in this connection are gelatin, casein, adestin, hemoglobin, egg albumin,  $\beta$ -lactoglobulin and clupein.

The titration curves of proteins are expressed in terms of  $pH$  and of  $h$ . The quantity  $h$  is simply the difference between the equivalents of HCl or of NaOH added to one mole of protein, and the equivalents of free  $H^+$  or of free  $OH^-$  present at the observed  $pH$ ; the starting point for the titration curve being the isoionic point of the protein. In the extreme acid or basic regions,  $h$  becomes a small difference between two large quantities and cannot be accurately measured; the range in which meaningful measurements can be made is from about  $pH$  1.5 to about  $pH$  12.  $h$  is equal to the net charge on the protein provided that the protein has not bound ions other than hydrogen ions.

In view of the dissociable groups available, the proton exchange of a protein may be expected to be concentrated in 3 zones centered about  $pH$  values 4, 7, and 10-12. The titration curve below  $pH$  6 is due almost entirely to the dissociation of carboxyl groups, whereas the segment between  $pH$  6 and  $pH$  8.5 is due to imidazole groups (histidine). That portion immediately above  $pH$  8.5 arises from the amino groups. The guanidine groups are so strongly basic that they should exist entirely in cationic form below  $pH$  11. There is evidence that the phenolic groups of proteins do not dissociate much below  $pH$  11. This leaves only carboxyl, amino, and imidazole groups to determine the whole sweep of the titration curve between  $pH$  2 and  $pH$  11.

<sup>33</sup> E. J. Cohn, *Physiol. Revs.* 5, 349 (1935).

The most important contributions to the subject of acid-base titration of proteins have been by Cannan and his group<sup>34</sup> and by Wyman<sup>35</sup> who studied the heat changes associated with the dissociation of oxyhemoglobin. Figure 8 shows the binding of hydrogen ions by egg albumin.

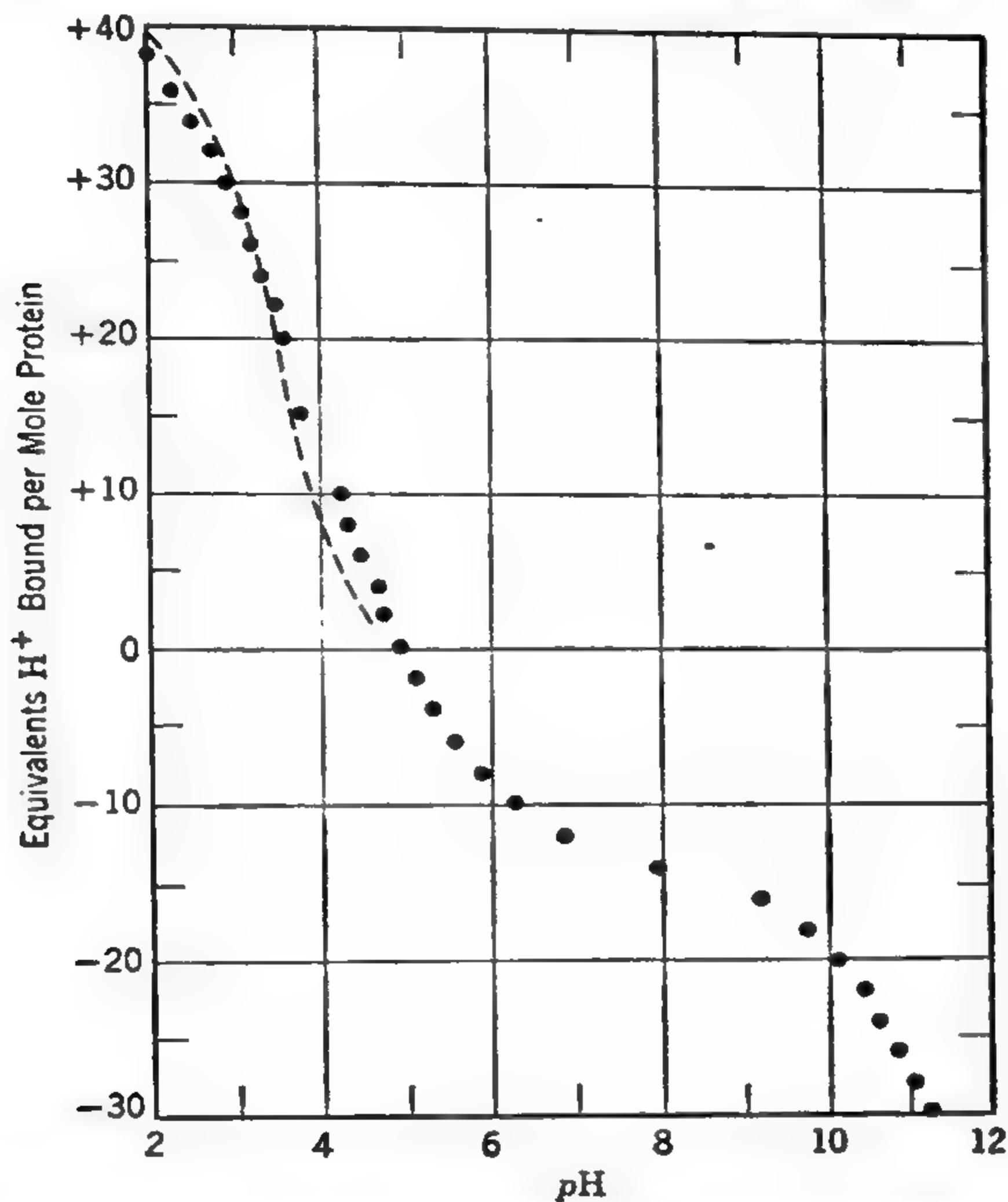


FIG. 8. Binding of  $H^+$  by egg albumin. At  $25^\circ C$ . 0.067 ionic strength. Dotted curve for single intrinsic ionization constant. (Data of Cannan.)

The study of the influence of temperature on the titration curve of proteins is very illuminating. Wyman calculates what he calls the apparent heats of dissociation by the relation

$$\Delta H = -2.303RT^2 \frac{d\text{pH}}{dT} \quad (53)$$

where  $d\text{pH}$  is the change in the  $\text{pH}$  of a solution of a protein in the presence of a constant amount of added acid or base with temperature. He then

<sup>34</sup> R. A. Kekwick and R. K. Cannan, *Biochem. J.* 30, 227 (1936).

R. K. Cannan, *Ann. N. Y. Acad. Sci.* 41, 243 (1941).

R. K. Cannan, *Chem. Revs.* 30, 395 (1942).

R. K. Cannan, A. H. Palmer, and A. C. Kibrick, *J. Biol. Chem.* 142, 803 (1942).

<sup>35</sup> J. Wyman, Jr., *J. Biol. Chem.* 127, 1 (1939).



plots the  $\Delta H$  values so calculated against  $pH$ , and, when such a plot is compared with the titration curve of the protein and with the known heats of dissociation of the individual groups, there remains little room for questioning the identity of the groups being titrated. Table 7 lists the groups along with their heats of dissociation.

TABLE 7

HEATS OF DISSOCIATION IN CALORIES PER MOLE OF GROUPS FOUND IN PROTEINS

Group	Amino Acid	$\Delta H$	$pK_a$
$\alpha$ -Carboxyl		-1,500 to 2,000	2.3
Carboxyl	Aspartic, glutamic	-1,500 to 2,000	3.6 to 4.3
Imidazole	Histidine	7,000	6.0
$\alpha$ -Amino	.....	9,000 to 12,000	9.6
$\epsilon$ -Amino	Lysine	11,000 to 12,000	10.5
Phenolic	Tyrosine	6,000	10
Guanidine	Arginine	12,000 to 13,000	12.5

A protein is a polybasic acid of great complexity, but the binding of protons does not differ in principle from the binding of any type of small ion, which will be discussed at greater length in Chapter 10. Two effects are to be noted: (1) the statistical effect and (2) electrostatic interaction. The statistical effect takes into account the probability of a proton being bound as a function of the total number of binding sites and of the binding sites already occupied. The electrostatic interaction involves a consideration of the work required to overcome electrostatic repulsion in placing a proton on the protein. These effects were considered by Linderström-Lang,<sup>36</sup> Cannan,<sup>34</sup> and by Klotz.<sup>37</sup>

If the only effect operating is the statistical one, the binding of ions can be expressed as a single ionization constant. This is entirely reasonable. Suppose that we have a solution of acetic acid of a given concentration. The behavior of the acetic acid can, as we have seen, be expressed by one ionization constant. Now, suppose that further we attach the acetic acid molecules to larger suspended particles without changing their ionization constant. If the acetic acid molecules are placed far enough apart so that they do not interfere with each other, we can evidently still describe the ionization of the acetic acid by one constant. Shown in Fig. 8 is a dotted curve expressing the ionization of 41 groups with a single ionization constant equal to  $4.0 \times 10^{-4}$ .

## ACID-BASE CATALYSIS

It is well known that hydrogen and hydroxyl ions catalyze the hydrolysis of esters. In fact, the rate of hydrolysis of sucrose by acids was used as a

<sup>36</sup> K. Linderström-Lang, *Compt. rend. trav. lab. Carlsberg* 15, No. 7 (1924).

<sup>37</sup> I. M. Klotz, *Cold Spring Harbor Symposia Quant. Biol.* 14, 97 (1950).

measure of their strengths before the advent of the modern methods for determining hydrogen-ion concentrations.

It soon became apparent, however, that hydrogen and hydroxyl ions were not alone in possessing catalytic powers towards esters. The substances that have such catalytic powers may be divided into two groups: (1) hydrogen ions, i.e.,  $\text{H}_3\text{O}^+$  ions, undissociated molecules of weak acids; and (2) hydroxyl ions, undissociated molecules of bases, and anions of weak acids. It can be seen, therefore, that the capacity to act as a catalyst in hydrolytic reactions is not limited to hydrogen and to hydroxyl ions but is possessed by all substances capable of accepting or donating protons. This conclusion is in keeping with Brønsted's definition of acids and bases.

It is believed that the hydrolysis of an ester involves a combination of the ester with water to form a complex. The proton acts as a catalyst by combining with this complex to produce an unstable molecule which then breaks down to yield the alcohol and acid. Thus, in order to have an acid-base catalyst, two types of substances must be present: an acid capable of supplying a proton, and a base capable of accepting a proton. Water being an amphiprotic solvent, it can either accept or donate protons.

In Table 8 are shown the velocity constants for the hydrolysis of ethyl acetate in the presence of various substances. All these substances are capable of either accepting or donating protons.

TABLE 8

VELOCITY CONSTANTS OF THE HYDROLYSIS OF ETHYL ACETATE  
IN THE PRESENCE OF VARIOUS ACID-BASE CATALYSTS

Catalyst	Velocity Constant
Hydroxyl ions	6.5
Hydrogen ions	$6.5 \times 10^{-3}$
Acetic acid (unionized)	$1.45 \times 10^{-6}$
Acetate ions	$2 \times 10^{-6}$
Water	Negligible

## PROBLEMS AND QUESTIONS

- Outline a method for the calibration of the pH scale.
- Derive the expression for the pH of a weak base in the presence of its salt of a strong acid.
- Calculate the approximate pH of a solution that has been obtained by mixing 100 cc. of a  $M/50$   $\text{K}_2\text{HPO}_4$  solution with 100 cc. of a  $M/40$   $\text{NaH}_2\text{PO}_4$  solution. What is the ionic strength of this buffer?  
*Ans.: pH = 7.11;  $\mu$  = 0.0425.*
- The potential of the saturated calomel half cell is +0.2443 volt. The measured e.m.f. against a hydrogen electrode is 0.305 volt. What is the pH of the solution at 25° C.?  
*Ans.: pH equals 1.08.*

## OXIDATION-REDUCTION

We shall limit ourselves in this short discussion to a consideration of electronic oxidation-reduction, attempting only to outline the general theory of such systems together with the experimental techniques involved in the use of indicators and electrometric methods. This will be followed by a brief summary of biological oxidations.

The general term "oxidation-reduction" is, in some respects, an unfortunate one. It is true that there are many reactions involving oxygen which come under this heading, for example.



On the other hand, many reactions that are termed oxidations do not involve oxygen, such as



In the above reaction, the ferrous ion has been "oxidized" to the ferric state although no oxygen has been used. Oxidation here involves the loss of an electron



In the same reaction chlorine was reduced; it gained an electron. We can generalize this experience. If any component of a system is reduced, there must be a simultaneous and equivalent oxidation of some other component. On the basis of the above example, we may formulate a general definition of an oxidation or of a reduction; an oxidation involves the loss and a reduction the gain of electrons. According to this definition, a metal in the presence of its ions is an oxidation-reduction system; i.e.



An example with which all biochemists are familiar and which we have already discussed is the hydrogen electrode. Here the hydrogen gas is the reduced and the hydrogen ions the oxidized form.



## ELECTRODE POTENTIALS

If an inert metal such as platinum is immersed in a reversible oxidation-reduction system such as a solution of ferrous and ferric chloride, and if this electrode is connected to a hydrogen half cell at unit activity of hydrogen ions, a potential difference will be observed. It can be shown theoretically and experimentally that the observed voltage is a function of the ratio of the oxidized to the reduced form; the more of the oxidized component, the more positive will be the platinum electrode in respect to the hydrogen electrode.

We wish to indicate how the relation between the measured potential difference and the ratio of the oxidant to the reductant can be derived. Consider again the reaction



We can formulate this reaction in the usual manner of chemical equilibria

$$\frac{\text{Fe}^{+++} \times (-)}{\text{Fe}^{++}} = K \quad (1)$$

If a platinum wire is immersed in this system, there will be a tendency for the electrons in solution to flow into the wire. On the other hand, the wire already has a concentration of electrons, and the direction of flow will be governed by the escaping tendencies of the electrons in the two phases. It can be shown that the work done in transferring an equivalent of electrons from the solution into the wire is

$$\text{Work} = RT \ln \frac{e_m}{e_s} \quad (2)$$

where  $e_s$  is the concentration of electrons in the solution,  $e_m$  is the electronic concentration in the metal,  $T$  is the absolute temperature, and  $R$  is the gas constant, equal to 1.99 calories per degree.

The work is equal to the quantity of electricity transferred multiplied by the potential at which the transfer is made. As we remember from previous considerations

$$\text{Work} = nFE \quad (3)$$

where  $n$  is the number of equivalents transferred,  $F$  is a conversion factor, and  $E$  is the potential at which the transfer is made. Combining equations 2 and 3 and rearranging, we have

$$E = \frac{RT}{nF} \ln e_m + \frac{RT}{nF} \ln \frac{1}{e_s} \quad (4)$$

where  $E$  is the oxidation-reduction potential.

Equation 1 is substituted in equation 4 by equating  $e$ , and  $(-)$ . The concentration of electrons in the metal ( $e_m$ ) is a constant. The first term on the right side of equation 4 is, therefore, a constant. Combining this constant with the constant in equation 1 to form a new constant  $K_1$ , we have

$$E = K_1 + \frac{RT}{F} \ln \frac{\text{Fe}^{+++}}{\text{Fe}^{++}} \quad (5)$$

When the concentration of ferric ions equals that of the ferrous,  $E$  equals  $K_1$ .  $E$  is usually written  $E_a$ , and  $K_1$  is denoted by  $E_0$ . Then

$$E_a = E_0 + \frac{RT}{F} \ln \frac{\text{Fe}^{+++}}{\text{Fe}^{++}} \quad (6)$$

This equation can be generalized in the form

$$E_a = E_0 + \frac{RT}{nF} \ln \frac{\text{Ox}}{\text{Red}} \quad (7)$$

At 30° C. and logarithm to the base 10, and for one electron transfer

$$E_a = E_0 + 0.06 \log \frac{\text{Ox}}{\text{Red}} \quad (8)$$

For a reduction involving two electrons at 30° C., the equation is

$$E_a = E_0 + 0.03 \log \frac{\text{Ox}}{\text{Red}} \quad (9)$$

The oxidation-reduction potential is always referred to the normal hydrogen electrode, which, as we have seen, is defined as having zero potential. In practice, it is generally more convenient to use the calomel half cell and correct for the potential contributed by the calomel half cell.  $E_0$  in equation 6 has considerable significance. As pointed out above, if the concentration of the oxidant equals that of the reductant, the term involving the ratio of oxidant to the reductant becomes zero and the observed voltage is equal to  $E_0$ .  $E_0$  is known as the standard oxidation-reduction potential and is used to compare oxidation-reduction systems with one another.

The oxidation-reduction potential is a measure of the tendency a substance has to give up or to take up electrons. Stated in a different way, it is a quantitative measure of the free energy of the oxidation of a substance. Evidently, in order to have a potential established at an inert electrode, there must be an electronic transfer, although with some reactions involving no such transfer there are certain tricks which can be resorted to and

which yield an apparent potential. It should be added that the oxidation-reduction potential is an intensity factor in the same sense as temperature is an intensity factor and does not in any way indicate the reducing or oxidizing capacity of a system.

Figure 1 shows the plot of the oxidation-reduction potential as a function of percentage of oxidant present.

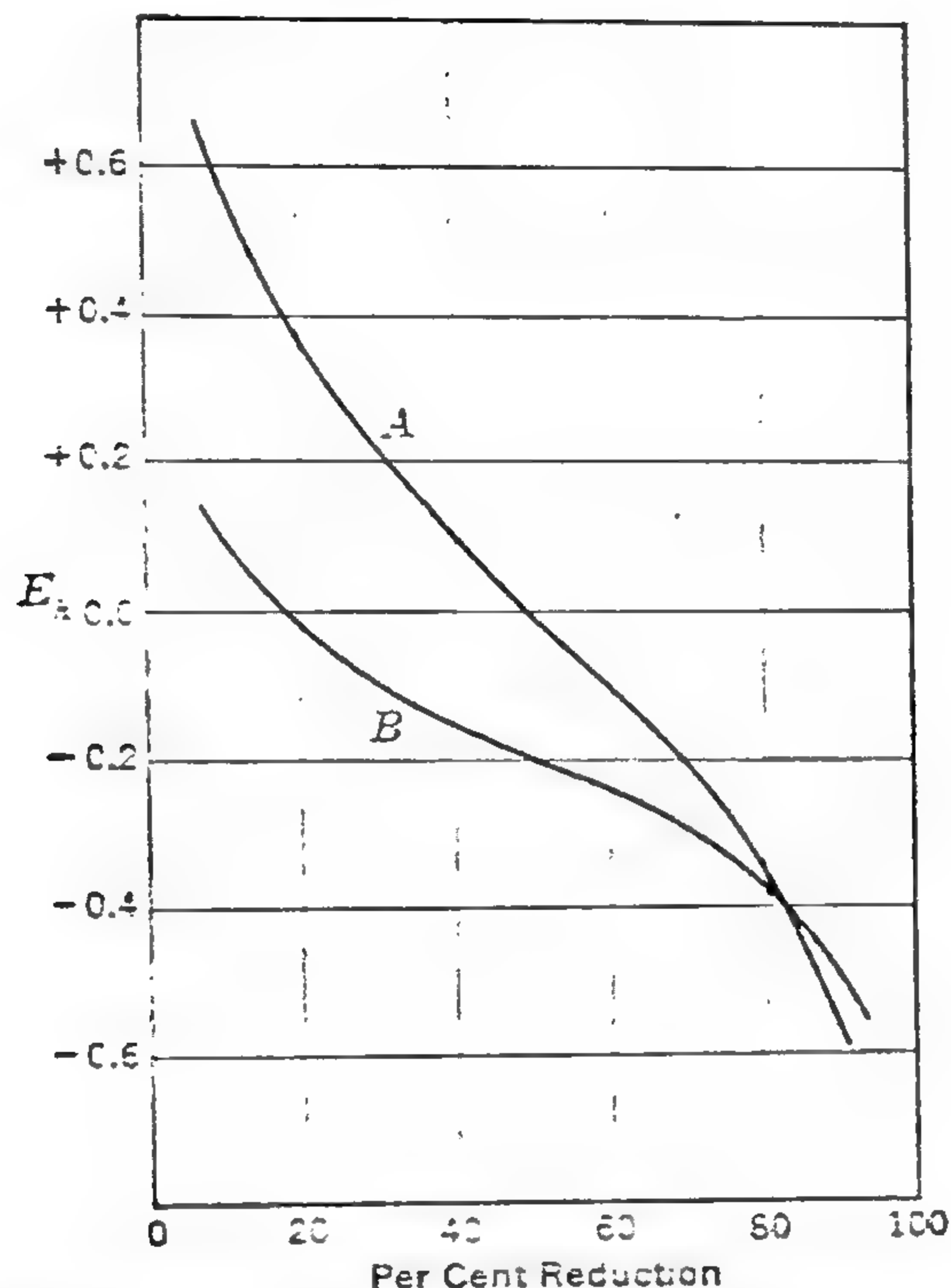


FIG. 1. Oxidation-reduction potential as a function of the percentage of oxidant present. System *A* involves the transfer of one electron; system *B* involves a two-electron transfer.

We see from Fig. 1 that, although system *A* has a higher normal oxidation-reduction potential than system *B*, if *B* contains 90 per cent oxidant *B* will tend to oxidize system *A* if that system has 90 per cent reductant and 10 per cent oxidant.

We have said nothing regarding the relative speeds of oxidation or reduction, and oxidation-reduction potentials are not capable of giving us any information on this point. Such potentials give us, in truth, a negative answer. We can say definitely that one system with a lower potential can never oxidize a system of higher potential in the same way as we can say that heat can never, without some external agency, flow from a cold to a hot body. We cannot, however, say that a system at a higher potential



will definitely oxidize a system of a lower potential. The reaction may not proceed, or, if it does, it may go so slowly that it is of no practical interest. Sometimes an empirical relation is observed between the speed of oxidation and the oxidation potential for a series of closely related compounds. Michaelis and Smythe<sup>1</sup> found that in a series of organic iron compounds the autoxidizability of the ferro compounds at a given pH closely paralleled the normal oxidation-reduction potential of the ferro-ferri systems of the compounds. The more negative the potential, the greater was the autoxidizability.

### EFFECT OF HYDROGEN IONS

The hydrogen-ion concentration of oxidation-reduction systems must usually be considered, because in most systems the reduced form can exist as an anion which can accept hydrogen ions and so become inoperative as far as contributing to the potential. Naturally, the potential of such a system is greatly influenced by the hydrogen-ion concentration.

A reduction involving the formation of an anion may be expressed as



and the electrode equation is

$$E_h = E_0 + \frac{RT}{F} \ln \frac{\text{Ox}}{\text{Red}^-} \quad (10)$$

but the reduced form ionizes as



and

$$\frac{\text{H}^+ \times \text{Red}^-}{\text{H Red}} = K \quad (11)$$

The total reduced form, Red, is equal to the ionized plus the unionized form

$$\text{Red} = \text{H Red} + \text{Red}^- \quad (12)$$

Combining equations 11 and 12, we have

$$\frac{\text{H}^+ \times \text{Red}^-}{\text{Red} - \text{Red}^-} = K \quad (13)$$

or

$$\text{Red}^- = \text{Red} \frac{K}{\text{H}^+ + K} \quad (14)$$

<sup>1</sup> L. Michaelis and C. V. Smythe, *J. Biol. Chem.* 94, 329 (1931).

Substituting equation 14 in equation 10, there results

$$E_h = E_0 + \frac{RT}{F} \ln \frac{\text{Ox}}{\text{Red}} \left( \frac{H^+ + K}{K} \right) \quad (15)$$

or

$$E_h = E_0 + \frac{RT}{F} \ln \frac{\text{Ox}}{\text{Red}} - \frac{RT}{F} \ln \frac{K}{H^+ + K} \quad (16)$$

If the ratio of the oxidant to the reductant is unity, equation 16 becomes

$$E_h = E_0 - \frac{RT}{F} \ln \frac{K}{H^+ + K} \quad (17)$$

If the value of  $K$  is very small as compared with the hydrogen-ion concentration, so that it can be neglected, we have

$$E_h = E_0 - \frac{RT}{F} \ln \frac{K}{H^+} \quad (18)$$

or

$$E_h = E_1 - \frac{RT}{F} \ln \frac{1}{H^+} \quad (19)$$

and at 30° C.

$$E_h = E_1 - 0.06 \text{ pH} \quad (20)$$

and the system could be used to determine the hydrogen-ion concentration.

The above example involving the formation of an anion with the transfer of only one electron is very unusual for organic compounds and was given for the sake of simplicity. The general rule is that the reduction of an organic compound involves the addition of two electrons, and it was thought for a number of years that these electrons had to be transferred simultaneously. Michaelis,<sup>2</sup> however, has pointed out that in several organic systems there is definite evidence for stepwise electronic transfer.

### STEPWISE OXIDATION

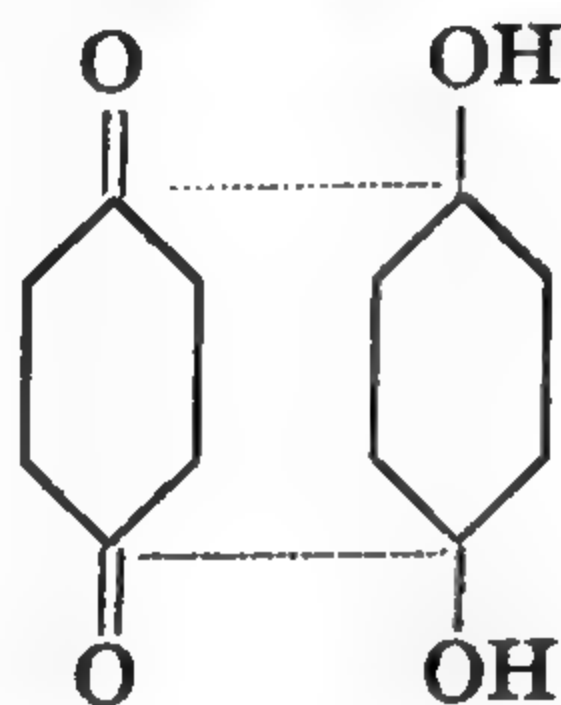
Michaelis cites three criteria for deciding whether the electronic transfer occurs in one or two steps: (1) the color of the intermediate compound, (2) the study of the magnetic moment, and (3) potentiometric oxidation titration curves. Before discussing these three criteria briefly we must fix clearly in mind what we mean by stepwise oxidation. Consider quinhydrone, for example.

<sup>2</sup> L. Michaelis, *C. 12 Spring Harbor Symposia Quant. Biol.* **L**, 224 (1933); **VII**, 33 (1939); *Chem. Rev.* **16**, 450 (1935).

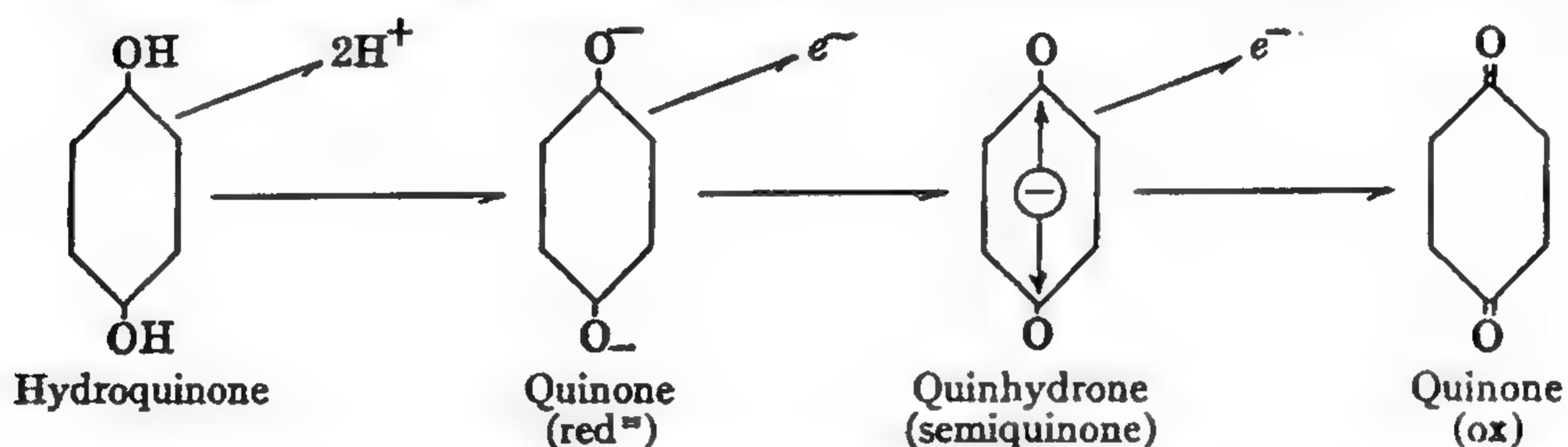
L. Michaelis and C. V. Snythe, *Ann. Rev. Biochem.* **7**, 1 (1938).

L. Michaelis and M. P. Schubert, *Chem. Revs.* **22**, 437 (1938).

As is well known, quinhydrone is an equimolecular mixture of hydroquinone and quinone. It is frequently used in a half cell to determine the hydrogen-ion concentration. It was first proposed that quinhydrone exists as a meriquinone, i.e.,



On the basis of a stepwise oxidation, we would postulate a semiquinone.



Quinhydrone, however, exists only in the solid state. In solution, it is completely dissociated into hydroquinone and quinone. A semiquinone is really a free radical and usually has a distinctive color. Its appearance in appreciable amounts can be detected by the color of the solution.

It is known that all molecules containing an odd number of electrons are paramagnetic, and those with an even number are usually diamagnetic. By determining the magnetic moment of a compound in solution, the presence of a free radical is detectable. The method has been used but seldom as it is limited by the solubility and stability of the free radical; the concentration of the semiquinone in solution must be high in order to measure the magnetic properties.<sup>3</sup>

The most useful method for studying the formation of semiquinones is by potentiometric oxidation titrations in which the completely reduced form is titrated with a convenient oxidizing agent and the oxidation-reduction potential measured. As we have seen for a univalent oxidation (30° C.)

$$E = E_0 + 0.06 \log \frac{\text{Ox}}{\text{Red}} \quad (21)$$

and for a bivalent oxidation

$$E = E_0 + 0.03 \log \frac{\text{Ox}}{\text{Red}} \quad (22)$$

<sup>3</sup> L. Michaelis, *J. Am. Chem. Soc.* 63, 2446 (1941).



If the oxidation occurs in two distinct univalent steps, the oxidation curve will be sigmoidal in this. Frequently, of course, there is considerable overlapping of the two oxidation steps which makes the analysis of the curve difficult. Figure 2 shows the titration of pyrogallous with ferricyanide. This is a clear example of two-step oxidation.

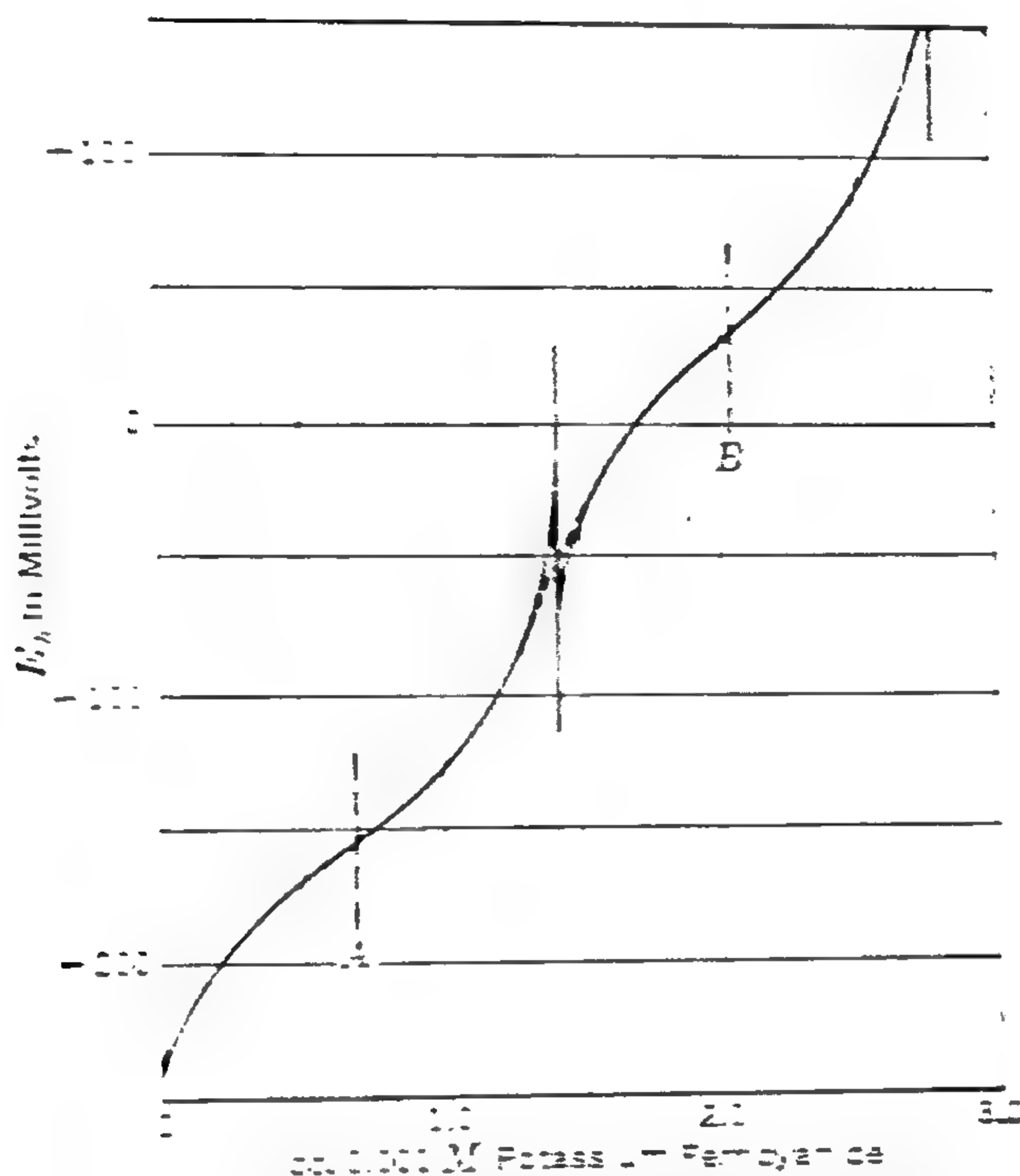


Fig. 2. Titration of pyrogallous acid with ferricyanide. A is the midpoint of the first electron transfer; B is that of the second. (Matheson.)

If the amount of the free radical form is large, the two steps of the titration are very conspicuous. On the other hand, if the amount of free radical is small, the two steps blend into one curve which is that of a bivalent oxidation. By a mathematical analysis of such a curve the amount of the free radical can be estimated.

Matheson has pointed out that the mathematical formulation of the titration of a radical by a reductant is closely analogous to that of the ionization of a weak acid. It has already been noted that the ratio of the forward and reverse ionization constants of a dibasic acid must be 4 to 1. Similarly, the ratio of the rate constants for the release and bound completely intermediate radical ions must be 4 to 1. This is purely a matter of statistical probability.

$$K_1/K_2 = k_1/k_2 = 4:1 \quad (14)$$

When the statistical effect is the only factor operating, the electron dissociation constants for a two-electron transfer also bear a ratio of 4 to 1 to each other. Under these circumstances, the oxidation-reduction titration curve will have only one inflection and the calculated constant for the electron dissociation will equal the square root of the product of the two individual dissociation constants.

Shaffer<sup>5</sup> has suggested that the ability of some dyes to catalyze certain oxidation-reduction reactions is related to this property of yielding electrons stepwise. It is known, for example, that the reaction between  $\text{Ti}^{++}$  and  $\text{I}_3^-$  is a slow one;  $\text{Ti}^{+++}$  can give up one electron but not two, and  $\text{I}_3^-$  can accept two but not one. If a small amount of dye which is capable of stepwise oxidation-reduction is added, the reaction between  $\text{Ti}^{++}$  and  $\text{I}_3^-$  is greatly accelerated. The dye can take electrons one at a time from  $\text{Ti}^{+++}$  and give them two at a time to  $\text{I}_3^-$ . Another example is the system thallous and thallic sulfate and ceric and cerous sulfate. Both the thallous-thallic and cerous-ceric systems give reversible, well-defined oxidation-reduction electrode potentials. The potentials obtained with these systems indicate that the thallous sulfate should almost completely reduce the ceric salt, and yet, when the two salts are mixed together in solution, no appreciable reaction takes place even when the mixture is boiled. However, if a small amount of manganese sulfate is added, the reaction proceeds rapidly.

Shaffer explains this in the following manner: the change of  $\text{Ce}^{+++}$  to  $\text{Ce}^{++}$  involves one electron transfer; the change from  $\text{Tl}^+$  to  $\text{Tl}^{++}$  involves the loss of two electrons. The reaction between  $\text{Ce}^{+++}$  and  $\text{Tl}^+$  would, therefore, require a three-body collision. As such collisions would be expected to occur very infrequently, the reaction between the thallous and ceric ions is a slow one. In the presence of  $\text{Mn}^{++}$  ions the ceric ions are reduced and the  $\text{Mn}^{++}$  ions are oxidized to  $\text{Mn}^{+++}$  in a stepwise manner through  $\text{Mn}^{++}$ . The  $\text{Mn}^{+++}$  ions promptly oxidize the  $\text{Tl}^+$  ions to  $\text{Tl}^{++}$  ions. The stepwise oxidation as formulated by Michaelis is of fundamental significance for the reaction kinetics of biological oxidations.

In the discussion of the influence of the hydrogen-ion concentration on the oxidation-reduction potential, we considered the formation of an anion. The oxidized form may, however, be a cation which is capable of accepting hydroxyl ions, or we may have the simultaneous destruction of a cation and the creation of an anion. These complications will not be dealt with in detail; suffice it to say that the oxidation-reduction potential usually varies in a highly characteristic manner with the hydrogen-ion concentration.<sup>6</sup>

<sup>5</sup> P. A. Shaffer, *J. Phys. Chem.* **40**, 1021 (1936); *Cold Spring Harbor Symposia Quant. Biol.* **VII**, 50 (1939).

<sup>6</sup> W. M. Clark, *Public Health Report*, Reprint 826, March 30, 1923.

The dependence of several oxidation-reduction systems on  $pH$  is shown in Fig. 3. The lines in the figure correspond to the following systems: (1) hypothetical oxygen electrode, (2) *o*-phenanthroline, (3) ferrous-ferric, (4) quinhydrone, (5) ferro-ferricyanide, (6) methylene blue, (7) calomel electrode, (8) indigo monosulfonate, and (9) hydrogen electrode.

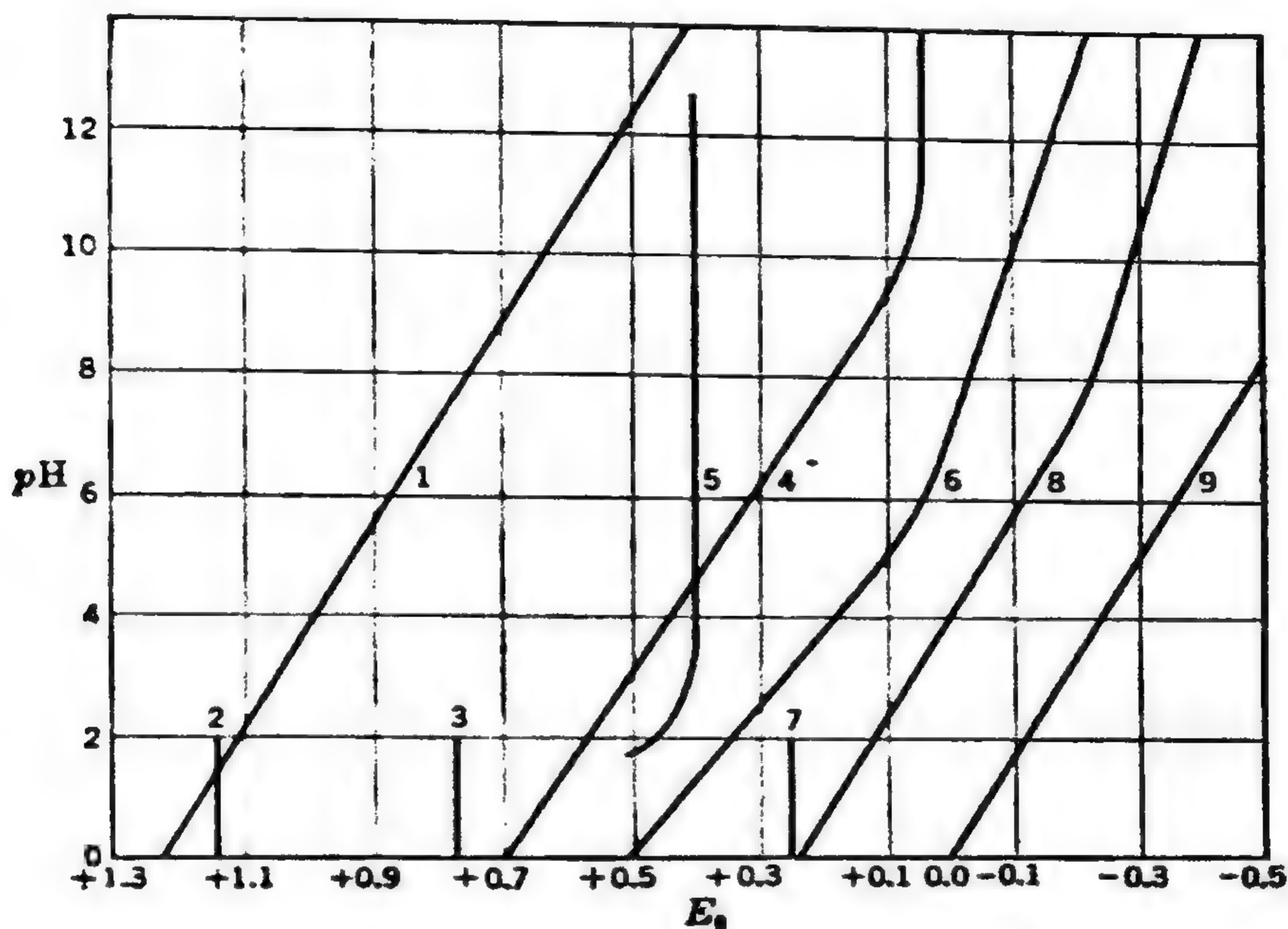


FIG. 3. Oxidation-reduction potentials as a function of  $pH$ .

The ionization constants of the oxidation-reduction systems are equal to the hydrogen-ion concentration at the inflection points of the curves (see Fig. 3); that is,  $pH$  equals  $pK$ . The sign of the change of the slope of the curve at the inflection point indicates whether the dissociation belongs to the oxidant or to the reductant. When the change in this slope is negative, this change is due to the ionization of the reductant; when positive, to the oxidant. It is also possible to evaluate the dissociation constants by simple acid-base titrations.<sup>7</sup>

A very fundamental analogy exists between acids and bases on the one hand, and oxidants and reductants on the other. According to Brønsted, the relation between the generalized acid A and base B is



<sup>7</sup> W. I. Hall, P. W. Preister, and B. Cohen, Paper XIV, *Public Health Report*, Supplement 71 (1928).



where (+) is a proton. We have already seen how oxidation-reduction can be formulated as



where (−) is the electron. Thus we see that the creation of an acid involves the addition of a proton to the base, whereas the creation of a reductant involves the addition of an electron to the oxidant.

To summarize this theoretical discussion, we may state that the oxidation-reduction potential depends on three conditions: (1) the innate tendency of a system to accept or yield electrons, (2) the ratio of the oxidant to the reductant, and (3) the hydrogen-ion concentration.

### MEASUREMENT

The oxidation-reduction potential can be determined by two methods, the indicator and the electrometric. For various reasons, indicators are less reliable in the measurement of the oxidation-reduction potential than for the measurement of *pH*.

It is no accident that a substance that undergoes oxidation and reduction easily is usually colored. Such a substance possesses loosely held electrons which can be transferred to another substance without difficulty; the vibrations of these electrons in the molecule are responsible for the color. Clark and co-workers and others have established a series of oxidation-reduction indicators whose potentials and ionization constants are known. By observing what indicators are oxidized or reduced by the change in color of the indicator, it is possible to assign to the system its approximate oxidation-reduction potential. The indicator method has proved of value in the study of living cells, where it has not as yet been possible to devise an electrode which would not injure the cell. Indicators are also useful in quantitative analysis in determining the end point of an oxidation-reduction reaction.

In determining the reducing intensity of cells with indicators, two methods are used. Either the dye is injected into the cell by a microinjection, or the dye is allowed to diffuse into the cell. The microinjection method cannot be used if the cells are small or sensitive to injury. In using the diffusion method, one has to be sure that the indicator will penetrate the cell. There seems to be a marked correlation between the reducing intensity of individual cells and a suspension of the same cells.

### ELECTROMETRIC METHOD

The electrometric method should be used whenever possible. As indicated above, the use of indicators is rather severely limited. The electrometric method is much broader and more exact in its application.

The apparatus for the purpose is quite simple. The quinhydrone electrode is such a common oxidation-reduction system that it seems needless to describe it. The same arrangement is employed to measure the potential of any oxidation-reduction system, unless the reductant is oxidized by the oxygen of the air, which necessitates a closed oxidation-reduction chamber through which an inert gas, such as nitrogen, is bubbled to sweep out all oxygen in the system. The nitrogen must be purified by passing it over heated copper filings or through pyrogallol solutions. The same technique is followed in the electrometric study of cell suspensions.

Unfortunately, a number of oxidation-reduction systems do not yield stable, well-defined potentials; indeed, some reactions such as the oxidation of an aliphatic aldehyde to the acid do not give a potential of any kind. If one insists upon measuring the potential of such a system, it will be found to fluctuate greatly and will, in general, depend on traces of oxygen or other impurities. Other reactions are sluggish and exhibit a stable potential only after several hours. Often the reasons for these difficulties are obscure. Two explanations for the sluggishness suggest themselves: (1) the material is irreversibly oxidized or reduced; (2) the oxidation-reduction is not of an ionic nature and cannot be expected to yield a potential. Perhaps the reason for the delay in the attainment of equilibrium in sluggish systems is that part of the reaction is not ionic but involves some internal change in the molecule. Ionic reactions, as a rule, proceed with extreme rapidity.

Conant and others have studied the irreversible oxidation-reduction of organic compounds and have developed methods for determining what they term the apparent oxidation-reduction potential. This is done by choosing some easily reversible system with the oxidant and reductant in equivalent amounts and using this in conjunction with an inert electrode. The substance under investigation is added, and the potential, which is due to the reversible system, observed. It is possible by this technique to bracket the irreversible potential between the potentials of two reversible systems.

Other methods have been followed. Some substances that do not of their own accord exhibit true potentials can be titrated with a reversibly reducible oxidant. For example, potassium ferricyanide has been used to titrate the reduced form of ascorbic acid. The ascorbic acid is oxidized and the ferricyanide is reduced. The observed potential is due to the ferri-ferrocyanide system, but, if sufficient time has been allowed for attainment of equilibrium, this potential must be equal to that of the ascorbic acid system and will continue to be so until all the reduced ascorbic acid has been oxidized.

Some systems are not reversibly oxidized or reduced until their molecules have been activated by an enzyme or perhaps by a dye. The reduction of



fumaric to succinic acid is reversible only in the presence of a succinic acid dehydrogenase and an oxidation-reduction indicator. The action of the indicator dye is probably that of a mediator; i.e., it takes the hydrogen which has been activated by the dehydrogenase and passes it on to the oxidant.

The important sulfhydryl ( $-\text{SH}$ ) systems have been carefully studied by Rykjan and Schmidt<sup>3</sup> who were able to show that under properly controlled conditions reversible and reproducible potentials can be obtained for the reaction,  $\text{RSSR} \rightleftharpoons \text{RSH}$ .

### BIOLOGICAL OXIDATION-REDUCTION

The measurement of oxidation-reduction potentials has not proved to be a very convenient tool for biological investigations. Much more fruitful has been the use of microrespirometers of the Warburg type. Such respirometers are designed to measure the oxygen uptake and the carbon dioxide production of tissue slices and of tissue homogenates. By the use of isotopic tracers, by isolation techniques, and finally by the use of microrespirometers a large amount of information has accumulated in respect to cellular metabolism. Most of this information is of a descriptive sort albeit on a chemical level; it does not as yet provide a field in which physical chemistry can be effectively applied on a broad scope.

In its simplest terms physiological oxidation consists in the removal of hydrogen from the substrate and the combining of it with molecular oxygen to form water or hydrogen peroxide. The living cell seldom, if ever, carries out this process in such a simple, direct fashion. Actually, the hydrogen is taken from the substrate and passed along in successive steps until it is finally combined with oxygen; as many as five steps may be involved. The cell releases the energy that it derives from oxidation somewhat as water is released through a series of locks in a canal. This gradual release of energy is more efficient and more easily controlled.

Four different types of substances are involved in cellular oxidation: dehydrogenases, hydrogen transports, oxidases, and peroxidases.

Dehydrogenases are specific enzymes which activate the hydrogen of the substrate so that the hydrogen can be removed from the substrate. The hydrogen transport systems convey the hydrogen removed from the substrate to the oxidase enzymes. The function of the oxidase enzymes is to activate the oxygen in the tissue so that it will quickly oxidize the hydrogen that is supplied by the hydrogen transports. The function of the peroxidases is to destroy the peroxides that may be formed. Carbon dioxide production involves the decarboxylation of the substrate.

<sup>3</sup> L. R. Rykjan and C. L. A. Schmidt, *Univ. Calif. Pubs. Physiol.* 8, 257 (1944).



There need not be an actual transfer of hydrogen atoms. For example, hydrogen atoms yield electrons to the cytochrome system and are thereby converted into hydrogen ions. The iron of the cytochrome system is reduced by the electrons, and the oxygen activated by the cytochrome oxidase accepts the electrons from the cytochrome system. The reduced oxygen then combines with protons to yield water.

Respiration can be either aerobic, in which event the ultimate hydrogen acceptor is molecular oxygen, or anaerobic and the hydrogen acceptor is some organic molecule. The body resorts to anaerobic respiration when the demand for oxygen exceeds its supply, i.e., in vigorous muscle activity or in malignant tumor. Anaerobic respiration is a type of fermentation and is sometimes known as glycolysis, the end product of which is, in the tissues of higher animals, lactic acid. The energy yield from aerobic respiration is very much larger than it is from anaerobic respiration.

It has become increasingly clear that amino acids, fatty acids, and sugars all enter a general metabolic pool which involves a series of di- and tricarboxylic acids and is known as Kreb tricarboxylic acid cycle. The portal of entry for the fatty acids is acetic acid, that for sugars is pyruvic acid, and that for amino acids is pyruvic acid, oxalacetic acid, and  $\alpha$ -keto-glutaric acid.

We have discussed in Chapter 2 the synthesis of high-energy phosphate bonds. It is probable that the main function of cellular respiration is the production of such phosphate bonds.

Two excellent books dealing with tissue metabolism are those by Baldwin<sup>9</sup> and by Lardy.<sup>10</sup>

## PROBLEMS AND QUESTIONS

1. Explain why hydrogen ions influence the oxidation-reduction potential of many systems and not of others.

2. What is meant by stepwise oxidation and what are the criteria for stepwise oxidation?

3. 10 cc. of 0.1 *M* FeCl<sub>3</sub> are mixed with 10 cc. of 0.1 *M* potassium ferrocyanide solution at 25°C. The oxidation-reduction potential of ferro-ferri cyanide is 0.36 volt and that of the ferro-ferri system is 0.771 volt. Calculate the concentration of the ferric ions after equilibrium is reached. What would be the oxidation-reduction potential of the mixture?

*Ans.:  $1.67 \times 10^{-5}$  molar;  $E_A = 0.566$  volt.*

<sup>9</sup> F. Baldwin, *Dynamic Aspects of Biochemistry*, The Macmillan Co., New York, 1947.

<sup>10</sup> H. A. Lardy, *Respiratory Enzymes*, Burgess Publishing Co., Minneapolis, 1949.

## ELECTROKINETICS

In general, when two surfaces come in contact there is a separation of electrostatic charges, one surface becoming negative in respect to the other. This separation of charges gives rise to a potential difference at the interface between the surfaces.

In 1808 Reuss<sup>1</sup> first showed that the passage of an electric current through a membrane of powdered quartz caused water to flow through the membrane. From this modest beginning have developed theories and techniques which have finally placed at the disposal of the biochemist the elegant tool of electrophoresis which has enabled him to study effectively complex mixtures of proteins.

It is important to realize that electrophoresis or the motion of charged particles in an electrical field is a particular manifestation of a more general phenomenon which has come to be known as electrokinetics and involves the motion of two charged surfaces relative to each other. The early workers centered their attention on attempts to calculate from experimental data the electrostatic potential between the charged surfaces. As we shall see, there are many ambiguities associated with such calculations and the emphasis has shifted towards a less formal approach.

The four methods available for the study of electrokinetics are basically similar in that all of them involve the relative motion of two surfaces in contact. These methods are:

1. Electroosmosis measures the rate of motion of a liquid relative to a solid under the influence of an external electrical field applied tangentially to the interface.

2. Streaming potential measures the potential resulting from the movement of a liquid relative to a solid in response to a mechanical force applied tangentially to the interface.

3. Electrophoresis or, as it was once called, cataphoresis involves the movement of a solid phase in respect to a liquid under the influence of an external electrical field.

<sup>1</sup> F. F. Reuss, *Mémoires soc. imperiale naturalistes Moscou* 2, 327 (1809).

4. The Zorn effect measures the electrical potential established by the contact of a solid phase in respect to a stationary liquid. The mechanical force is usually controlled by gravity, and this technique is sometimes known as the sedimentation potential method.

The study of electrophoresis has proved to be more rewarding than that of the other techniques, and we shall discuss electrophoresis at some length, briefly summarizing the methods and results of the other techniques.

## THE ZORN EFFECT

Smoluchowski<sup>1</sup> first derived the expression for the motion of charged particles in an electric field. It is evident that a charged particle suspended in a liquid and subject to an electric field will move towards the electrode of opposite charge. The resistance to motion in the liquid is given by Newton's law of flow

$$\text{Resistance to motion} = \eta \frac{du}{dl} \quad (1)$$

where  $\eta$  is the coefficient of viscosity of the liquid,  $u$  is the linear velocity of flow of the liquid, and  $l$  is the distance normal to the particle surface and extending into the liquid. The differential  $du/dl$  is thus the flow gradient normal to the surface of the particle. If the velocity of flow varies directly with the distance, we can replace  $du/dl$  by the ratio  $u/l$ . The electrical force acting on 1 square centimeter of the particle-liquid surface is

$$\text{Electrical force} = E\sigma \quad (2)$$

where  $E$  is the potential gradient of the external electrical field parallel to the surface of the particle and  $\sigma$  is the charge per unit area at the particle-liquid surface. The electrical force is the force tending to produce the motion of the particle; at a constant particle velocity the acting forces must equal the resisting forces, and

$$E\sigma = \eta \frac{u}{l} \quad (3)$$

Now if we assume that the particle is very large in respect to distance of separation of the charges at the surface, then we can treat the electrical field as that of a plane plane condenser. For such a condenser the relation between charge and potential is

$$\sigma = \frac{4\pi d\epsilon}{D} \quad (4)$$

where  $\sigma$  is the potential across the solid-liquid interface,  $d$  is the distance between the centers of gravity of the charges in the liquid and that on the solid.

<sup>1</sup> *Ann. Physik*, vol. 17, 1903, 182.



solid, and  $D$  is the dielectric constant. The motion that results arises from the electrical charges, and, accordingly, we can equate the mechanical and the electrical thickness of the double layer. This permits us to substitute equation 4 into equation 3, obtaining

$$u = \frac{\zeta DE}{4\pi\eta} \quad (5)$$

where  $\zeta$  is the potential difference across the double layer in electrostatic volts,  $E$  is the potential gradient in electrostatic volts per centimeter,  $\eta$  is the coefficient of viscosity in poise, and  $u$  is the mobility in centimeters per second. Remembering that one electrostatic volt is equal to 299.8 ordinary volts, we find that

$$u = 8.89 \times 10^{-6} \frac{\zeta DE}{\eta} \quad (6)$$

where  $u$  is now expressed in microns per second,  $\zeta$  in ordinary millivolts, and  $E$  in ordinary volts per centimeter. In reference to water at 25° C. equation 6 becomes

$$u = 0.0778\zeta E \quad (7)$$

The equation of Smoluchowski was derived on the basis of the following four assumptions: (1) the usual hydrodynamical equations for the motion of a viscous fluid may be assumed to hold both in the bulk of the liquid and within the double layer; (2) the presence of the colloidal particle produces a distortion of the electrical field in such a way that the electrical current passes tangentially along the surface of the particle; (3) the electrical double layer is so thin that the electrical field can be considered to be parallel to the double layer at all points; (4) the electrical field does not deform the double layer.

If the colloidal particle is very small and the electrical double layer is fairly thick, the third assumption of Smoluchowski's is certainly not valid. Henry<sup>3</sup> has taken account of this situation. He finds that, if the ratio of the radius of a spherical non-conducting particle to the thickness of the double layer is not less than 300, Smoluchowski's equation should hold within 1 per cent. As the ratio becomes smaller, the coefficient 4 in Smoluchowski's equation increases to a limiting value of 6, which is reached when the ratio is reduced to 0.5. If the particle is small enough and the thickness of the double layer large enough, the particle approaches the condition of an isolated charged sphere, in which event its electrophoretic mobility may be treated in the following simple manner. The force-producing migration is evidently  $QE$  where  $Q$  is the charge on the particle and  $E$  is the potential

<sup>3</sup> D. C. Henry, *Proc. Roy. Soc. (London)* A133, 106 (1931).

gradient of the external field. The resistance to motion is the Stokes factor  $6\pi r\eta u$ , where  $r$  is the radius of the sphere and  $u$  is its velocity through the medium. At a steady state these two factors must be equal, and

$$QE = 6\pi r\eta u \quad (8)$$

The potential of a charged sphere is

$$\zeta = \frac{Q}{Dr} \quad (9)$$

Substituting equation 9 into equation 8, we find

$$\zeta = \frac{6\pi\eta u}{DE} \quad (10)$$

We see from these simple considerations that, in the limit, the coefficient 4 in Smoluchowski's equation should become 6. This is in accord with the conclusions of Henry. Henry also concludes that, for non-conducting particles whose size is large in comparison with the thickness of the double layer, the electrophoretic velocity is independent of the size and shape of the particles and that Smoluchowski's equation is obeyed. If, however, the thickness of the double layer is comparable with the radius of the particle, the electrophoretic velocity becomes a function of both the size and the shape of the particles. Protein molecules, in general, fall in this size range; accordingly, their mobilities should be dependent on their size and shapes.

Henry<sup>4</sup> has also called attention to still another complicating factor, i.e., to surface conductance around the particle. That is, owing to the accumulation of ions in the electric double layer which surrounds the particle, the conductance in the immediate neighborhood of the particle is greater than that of the liquid in bulk. The effect of surface conductance is to reduce the potential gradient acting on the particle and leads to a reduction of the observed mobility.

#### MEASUREMENT OF ELECTROPHORETIC MOBILITY

Electrophoretic mobilities can be measured by two methods. One is to observe the motion of the particles directly with a microscope; this is called the micromethod. The other, which is used with particles too small to be seen with a microscope, employs the moving boundary of the colloidal solution. The colloidal solution is placed in a U tube, and the motion of the boundaries that the colloidal solution forms with an ultrafiltrate of the colloidal solution is observed.

In the micromethod a suspension of microscopically visible particles is placed in a thin glass cell, a difference of potential is established across the

<sup>4</sup> D. C. Henry, *Trans. Faraday Soc.* **44**, 1021 (1948).

ends of the cell, and the motion of the particles is observed with a microscope. There is an electroosmotic flow of liquid along the surface of the glass cell, but since the cell is a closed system this liquid must return through the center of the cell. The result is that near the top and the bottom of the cell there are stationary levels where the liquid is motionless. At these levels and only at these levels the observed mobility of the particles is their true speed in respect to the liquid. At all other levels the observed speed is the resultant of the electrophoretic and the electroosmotic speeds. If the ratio of the width of the rectangular electrophoretic cell to its thickness is great enough, the stationary levels lie at 0.211 and 0.789 fractions of the total cell thickness as demanded by the theory of Smoluchowski.<sup>5</sup>

As the ratio of the cell width to thickness diminishes, Komagata's<sup>6</sup> correction must be applied, namely,

$$\text{Stationary levels} = 0.5 \pm 0.2887 \sqrt{1 + \frac{1.255}{A}} \quad (11)$$

where  $A$  is the ratio of the width of the cell to its thickness.

If the mobilities at various levels through the cell are observed and these mobilities are plotted against the distance from the ceiling of the cell at which the mobility measurements were made, a hyperbolic curve is obtained. Such a curve is shown in Fig. 1.

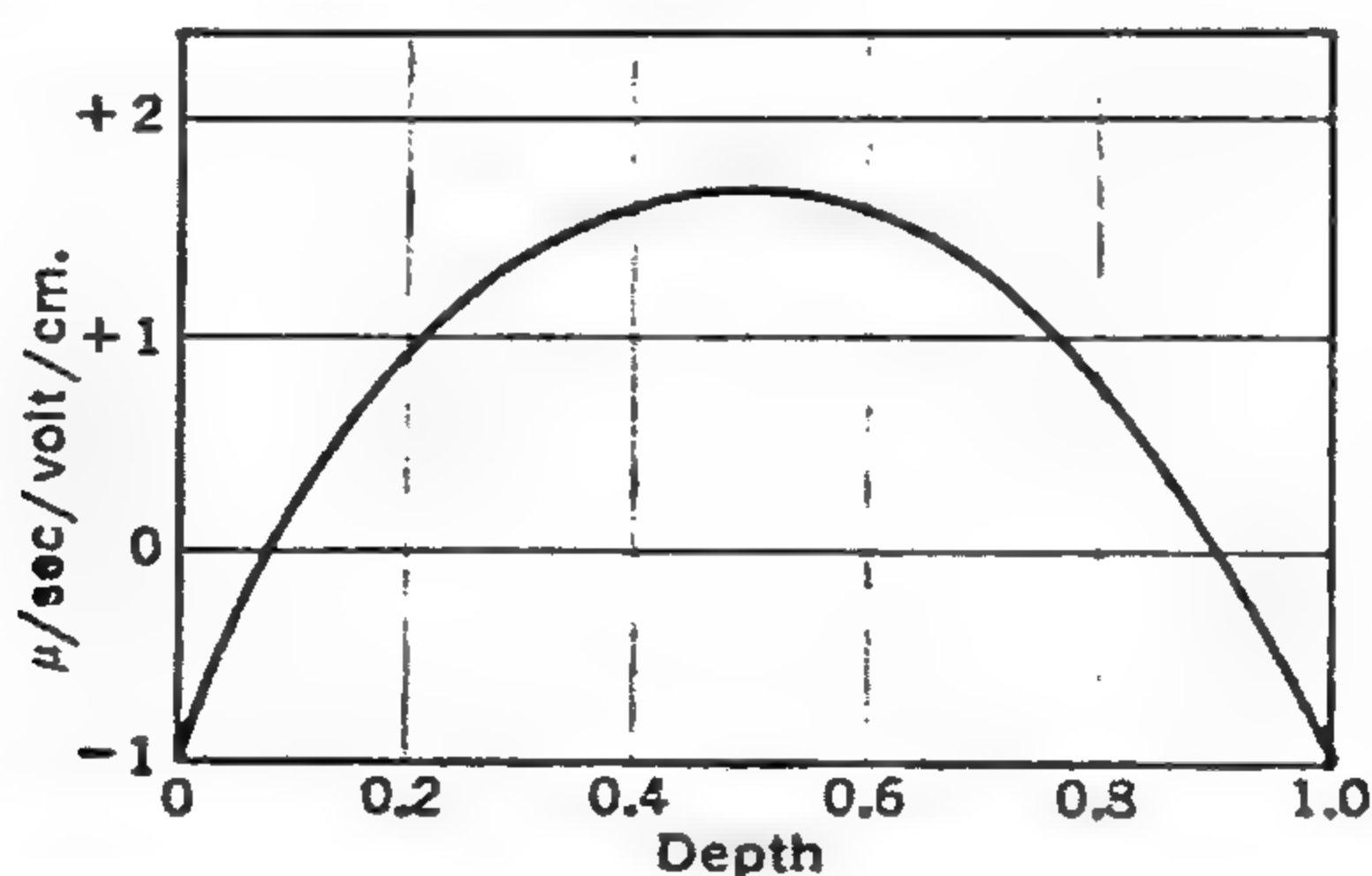


FIG. 1. Plot of the mobilities in a rectangular electrophoretic cell against the distance from the ceiling of the cell. The thickness of the cell is taken as unity.

Moyer<sup>7</sup> has described in detail the apparatus and methods for the microdetermination of electrophoretic mobilities. The construction of the rectangular cell is a job for an expert glass blower. The Abramson<sup>8</sup>

<sup>5</sup> M. v. Smoluchowski, *Handbuch der Elektrizität und der Magnetismus*, Vol. 2, p. 306, Leipzig, 1921.

<sup>6</sup> S. Komagata, *Researches Electrotech. Lab. Tokyo* No. 348 (1933).

<sup>7</sup> L. S. Moyer, *J. Bact.* 31, 531 (1936).

<sup>8</sup> H. A. Abramson and E. B. Grossman, *J. Gen. Physiol.*, 14, 563 (1931).



is perhaps to be the best of the many cells proposed. Briggs<sup>3</sup> has given a description of making a microcell that he reports is satisfactory. The author is not in favor of the use of the cylindrical microelectrophoretic cell because of a number of serious objections and, in spite of its simplicity, it is not acceptable.

The micro method has much to recommend it. It is comparatively inexpensive and measurements are made quickly and easily. The book by

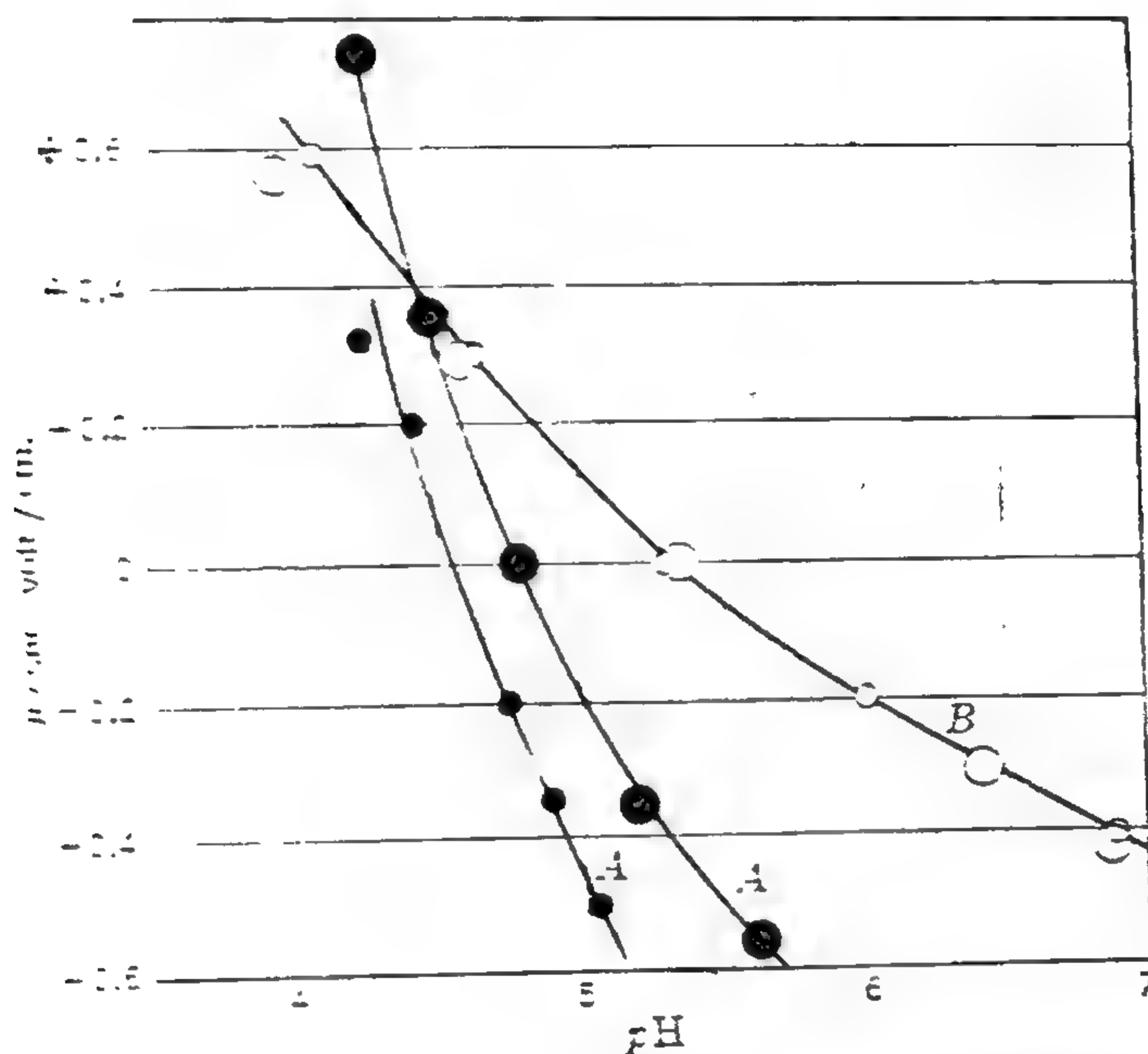


Fig. 1. Mobility vs. pH for (A) lysozyme (small circles) and adsorbed protein (large circles); (B) Hrs pseudoglobulin. (Meyer.)

the book by Meyer and G. H. G. should be consulted for a description of the method and the various substances studied by this method. A development that has been made in the use of the micro method was the discovery that the surface of various substances as glass, carbon, silica, and oil droplets become coated with the protein. The mobility of the protein solution becomes coated with the protein. The mobility of the protein solution is very close to, if not identical with, the mobility of the protein as determined with the moving-boundary method. This is a very important comparison of the moving-boundary mobility

<sup>3</sup> Briggs, J. H., *J. Biol. Chem.* 12, 73 (1940).

<sup>4</sup> Briggs, J. H., *J. Biol. Chem.* 32, 193 (1958); 37, 225 (1959).

<sup>5</sup> Meyer, R. H., and M. H. G. *Electrophoresis of Proteins*, Reinhold Publishing Co., New York, 1942.

ties with the mobilities of coated particles as determined with the micromethod.<sup>12</sup>

The micromethod is a splendid tool for the determination of the isoelectric point of proteins. The isoelectric point is a physical constant of great utility in protein chemistry. At this point the net charge on the particle is zero, and a protein has its minimum solubility at this  $pH$ . Microscopically visible glass particles are placed in a fairly concentrated solution of the protein (1 or 2 per cent) and become completely coated with protein in the course of a few minutes. The suspension of glass particles is then diluted with a convenient buffer, the dilute suspension is placed in a microelectrophoresis cell, and the mobility is determined. A series of buffers of different  $pH$  values are used, and the mobilities of the protein-coated particles are plotted against  $pH$ . The best line is drawn through these points and the line interpolated to zero mobility. The  $pH$  at which the mobility is zero is the isoelectric point of the protein. For example, the isoelectric point of pseudoglobulin used in the experiments shown in Fig. 2 is at  $pH$  5.3.

#### MOVING-BOUNDARY METHOD

The moving-boundary method employs a glass tube in the form of a letter U which contains the colloidal solution under investigation. The colloidal solution, for example protein, is placed in the lower part of the U tube and is layered over with buffer of the same electrolyte content as the protein solution. The proper electrolyte content of the buffer is obtained by ultrafiltering the buffered protein solution and using the ultrafiltrate to layer the protein solution, or more conveniently by making up the buffer to as nearly the same electrolyte content as the buffered protein and then dialyzing the protein solution against the buffer until equilibrium is reached. Sufficient electrolyte must be present to render the Donnan effect unimportant. The arms of the U tube are connected to non-polarizable electrodes, and the motion of the protein-buffer boundaries is observed under the influence of a known potential gradient. If the protein is colored, mobilities can be observed directly. If the protein is uncolored, the boundaries can be observed with ultra-violet light since proteins fluoresce in ultra-violet light, or the boundaries can be photographed since proteins absorb ultra-violet light. This necessitates the use of a quartz U tube as glass also absorbs ultra-violet light. A number of cells of the U tube type have been described. The cell used by Davis and Cohn<sup>13</sup> appears satisfactory.

Tiselius<sup>14</sup> has greatly improved the moving-boundary method, and his, along with the contributions of others, has made this technique into a very

<sup>12</sup> L. S. Moyer, *J. Phys. Chem.* 42, 71 (1938); *J. Biol. Chem.* 122, 641 (1938).

<sup>13</sup> B. D. Davis and E. J. Cohn, *J. Am. Chem. Soc.* 61, 2092 (1939).

<sup>14</sup> A. Tiselius, *Trans. Faraday Soc.* 33, 524 (1937).

powerful tool for the investigation of proteins and other biocolloids. It has become possible to analyze a complex mixture of proteins, to determine their electrophoretic mobilities, and, finally, under favorable conditions to obtain samples of the pure components. The improvements have been so considerable that it is substantially a new method, and it is customarily referred to as the Tiselius method. The U tube of Tiselius is divided into several sections which are fitted to each other by ground-glass joints that

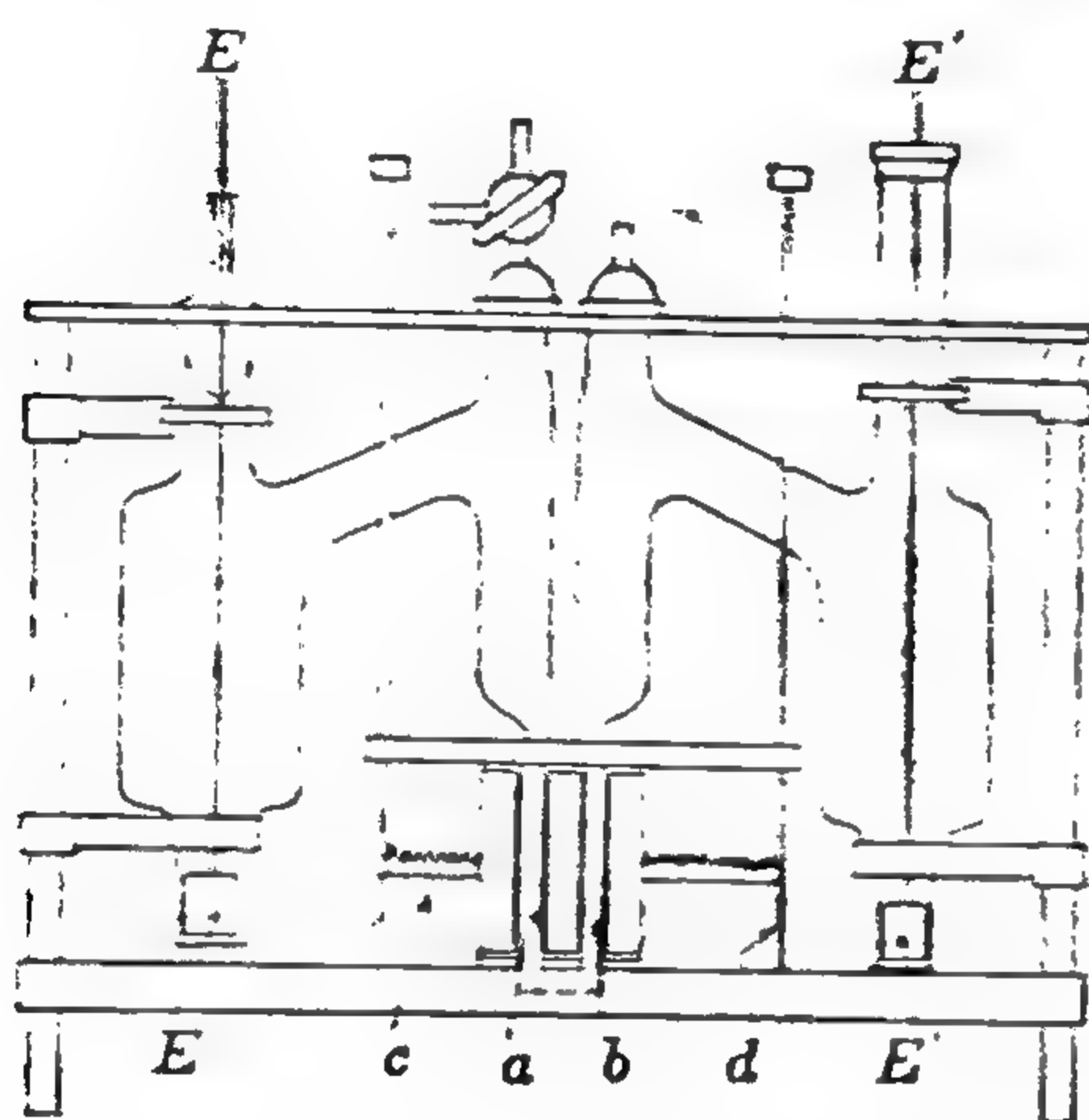


FIG. 3. The electrophoresis cell of Tiselius for quantitative study of the moving boundary and isolation of protein components.  $E$  and  $E'$  are silver-silver chloride electrodes.  $a$  and  $b$  are sections of the electrophoresis U tube.  $c$  and  $d$  are mechanical devices for moving sections  $a$  and  $b$ .

can be slid into and out of place by a mechanical device. The movement of the protein fractions is watched, and when a certain fraction is located in a particular section of the U tube this section is moved out and the contents isolated. As in the simple U tube, the protein solution is in the bottom half of the U. Over the protein solution is layered the buffer, whose electrolyte concentration is the same as that of the protein solution. Sharp boundaries between the protein solution and the buffer must be obtained.

Figure 3 shows a Tiselius electrophoresis cell and holder as modified by other workers. The most important improvement that Tiselius instituted was the method of observing protein boundaries. He used the so-called schlieren or shadow method. When light passes through a boundary separating two liquids of different indices of refraction, the light is refracted or bent in the direction of the liquid of greater refractive index. It is a common observation, for example, that a straight elongated object sticking into water appears bent to an observer. The buffer solution and the protein solution have different indices of refraction, the difference being proportional to the protein concentration; the greater the protein concentration, the greater will be the bending of the light rays that pass through the protein-buffer boundary. If an image of the electrophoresis cell is focused on a ground-glass plate and a knife edge is placed in the beam of light emerging from the electrophoretic cell in such a way as to intercept the deflected beam, the boundary of the protein-buffer will appear opaque and, accordingly, the position of the boundary can be accurately located on the ground-glass plate as a dark band. This optical system is diagrammed in Fig. 4.



If the protein solution contains more than one electrophoretically distinct component, boundaries for each component will appear as the components move away from each other as the result of their different electrophoretic mobilities, and these boundaries can be visualized on a ground-glass plate. Although the vertical width of the dark schlieren bands is proportional to the protein concentration, no accurate idea of the relative concentrations of the components can be conveniently achieved by this method.

Two separate methods have been developed which enable the boundaries to be located and a quantitative estimate of the components present to be

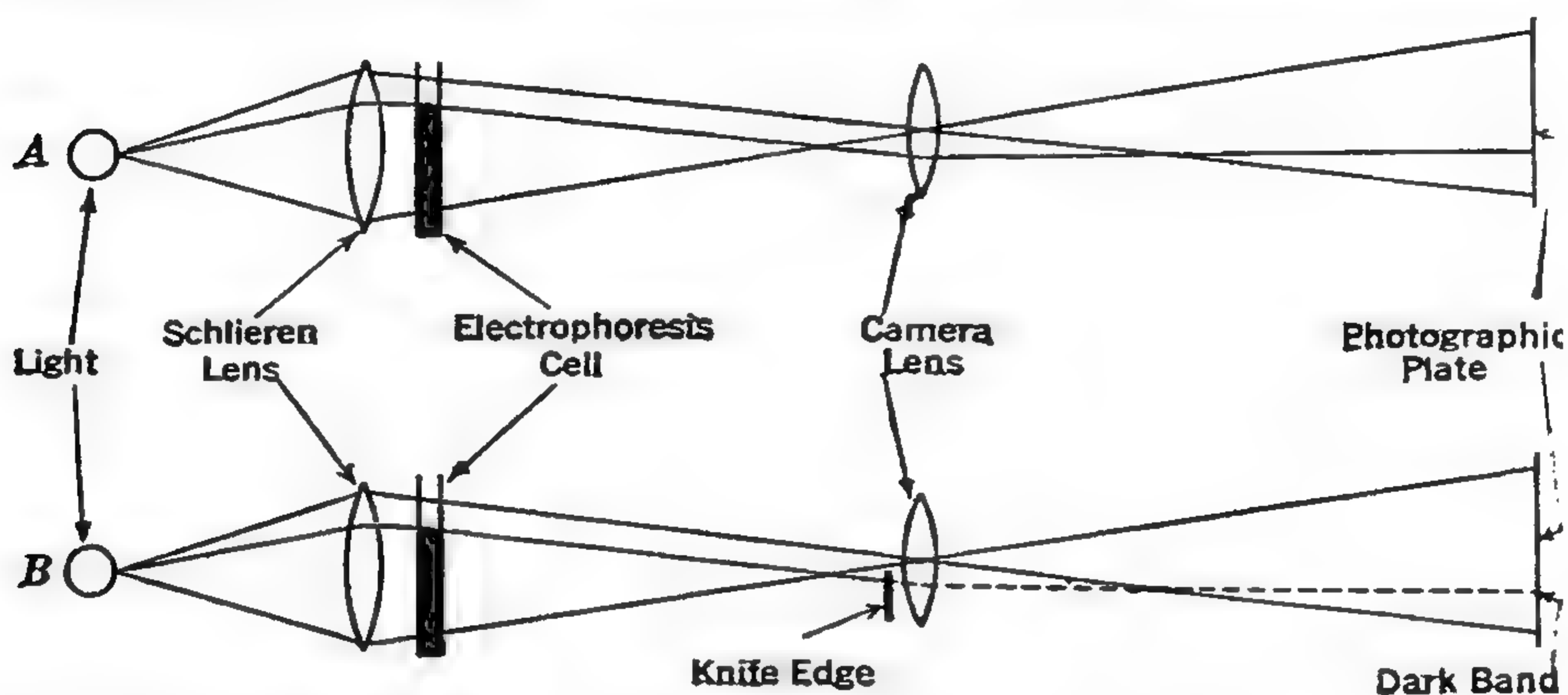


FIG. 4. Schlieren method for visualizing the position of a boundary in an electrophoresis cell. A, without knife edge. B, with knife edge in place.

made. These two techniques are the scanning method of Longworth and the cylindrical lens system of Philpot and Svensson.

Following the lead of Thorvert, Philpot<sup>15</sup> has developed an ingenious optical arrangement which allows not only the boundaries to be accurately located, but likewise a good estimate to be made of the relative concentrations of the protein components. If the proper information is available, the absolute protein concentrations also can be determined. The Philpot optical system has been studied and improved by Svensson.<sup>16</sup> The optics of this system is somewhat complicated; fortunately, the optics does not have to be understood to be used. The light source, a narrow horizontal slit, is focused on an oblique slit by means of a schlieren lens. The electrophoresis cell is placed as close to the schlieren lens as is practicable. The undeviated beam falls at the top of the oblique slit; the deviated beam falls lower down, and its deviation is proportional to the protein concentration. The light that suffers a downward displacement emerges from the oblique slit with a

<sup>15</sup> J. Thorvert, *Ann. Physik* (9), 2, 369 (1914).

J. St. L. Philpot, *Nature* 141, 283 (1938).

<sup>16</sup> H. Svensson, *Kolloid-Z.* 87, 181 (1939).

horizontal displacement relative to the undeviated beam, since all other components of the beam have been blocked by the inclined slit. Furthermore, the horizontal displacement is proportional to the original downward displacement. A camera lens collects the light from the oblique slit and

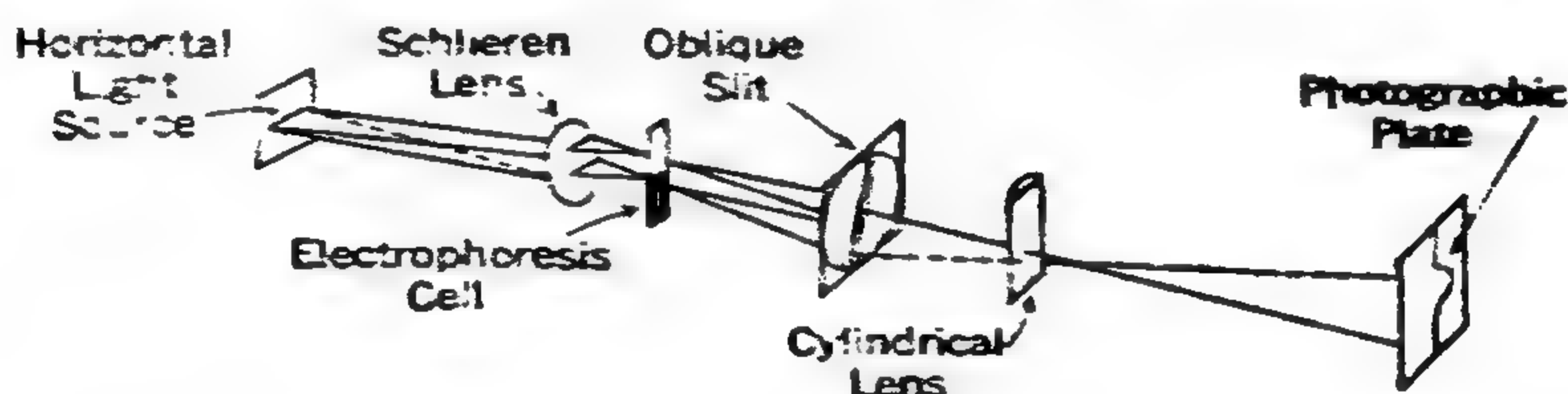


FIG. 5. Diagrammatic sketch of the cylindrical lens system for electrophoresis.

passes it into a cylindrical lens. The cylindrical lens has the property of focusing light horizontally but not vertically, and, if there is no concentration gradient in the electrophoresis cell, a sharp vertical straight line will be observed on the ground-glass plate. In the presence of a concentration gradient, the line on the plate will be shifted horizontally to produce a hump at this point. Since the deviated beam has undergone a horizontal displacement due to the blocking by the oblique slit and the deviation is proportional to the protein concentration, the size of the hump will be proportional to the protein concentration. The optical arrangement for the cylindrical lens is shown in Fig. 5.

It is necessary to show how the area under the curve that appears on the ground-glass plate or photographic plate is related to protein concentration. Figure 6 is a front view of the oblique slit.

Consider a point in the electrophoresis cell with the coordinate  $X_1$  and the index of refraction gradient  $n(X_1)$ . The deviation of the horizontal slit image passing through this point is

$$\Gamma_1 = c(n(X_1)) \quad (12)$$

where  $c$  is the thickness of the cell and  $l$  is the distance of the cell from the oblique slit. The horizontal component of the displacement of the light produced by the oblique slit is equal to  $\Gamma_1 \tan \theta$ . The coordinates of a deviated point of light on the ground-glass plate at unit magnification are then

$$X = X_1 \quad \text{and} \quad Y = \Gamma_1 \tan \theta \quad (13)$$

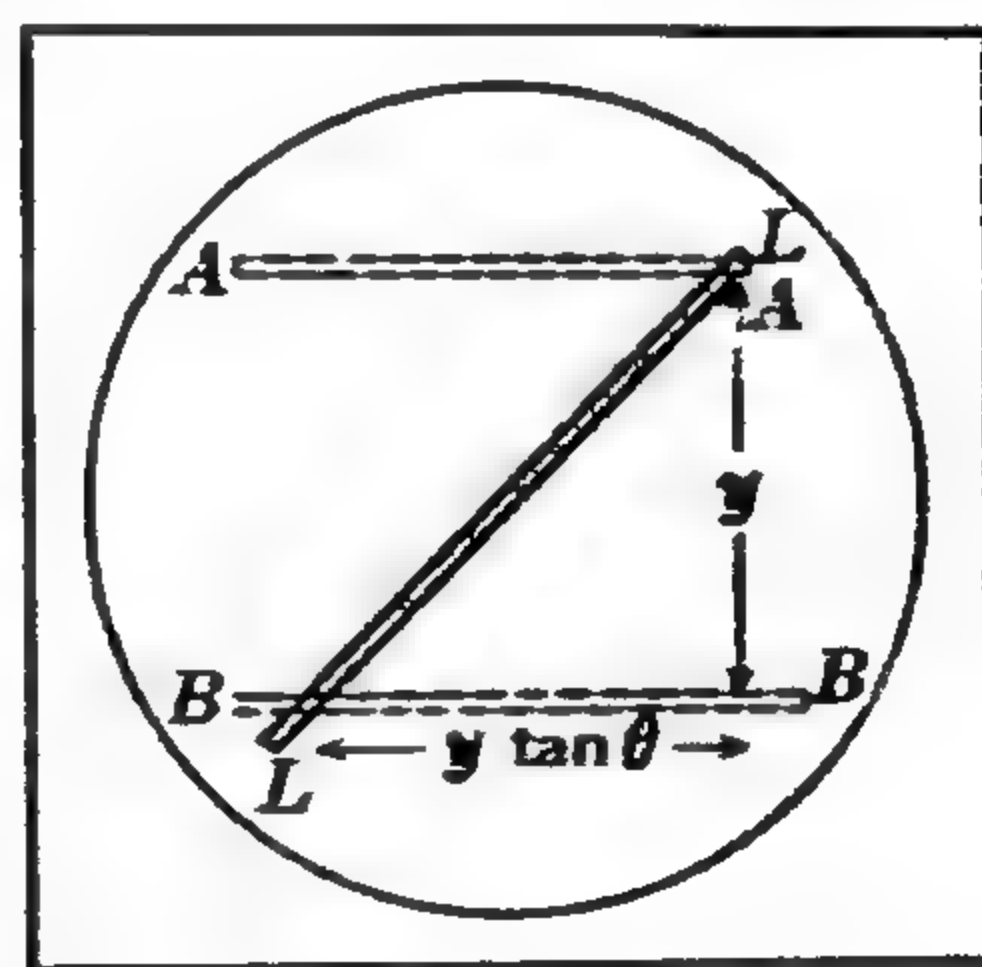


FIG. 6. Front view of the oblique slit.  $LL$  is the slit opening.  $AA$  is the position of the image of the undeviated slit.  $BB$  is the position of the deviated horizontal slit image due to concentration gradient.

The equation of the line formed by the points will be

$$Y = al \tan \theta \frac{dn}{dX} \quad (14)$$

which when rearranged becomes

$$dn = \frac{Y dX}{al \tan \theta} \quad (15)$$

Integrating equation 15 between the limits  $X_2$  and  $X_1$ , we have

$$n = \frac{1}{al \tan \theta} \int_{X_1}^{X_2} Y dX \quad (16)$$

Since there is a linear relation between the index of refraction and protein concentration

$$n = KC \quad (17)$$

where  $C$  is the protein concentration in per cent and  $K$  is a constant. Then

$$C = \frac{1}{Kal \tan \theta} \int_{X_1}^{X_2} Y dX \quad (18)$$

The integral  $\int_{X_1}^{X_2} Y dX$  is plainly the area under the curve; if the oblique slit is inclined at  $45^\circ$  to the horizontal,  $\tan \theta$  is unity and, accordingly,

$$C = \frac{\text{Area}}{K_1} \quad (19)$$

where  $K_1$  is a constant involving  $K$ ,  $a$ , and  $l$ . The area under the curve is obtained with a planimeter. If  $K_1$  is known, the absolute concentrations of the various components can be calculated; if  $K_1$  is unknown, the relative concentrations can be obtained by comparing the areas under the curves of the various protein components present.

Many technical details are involved in the construction and operation of the moving-boundary electrophoretic equipment. One important feature is that the water bath in which the electrophoresis cell is immersed must be maintained at or near the temperature of maximum density of the buffer in order to diminish the convection currents in the cell due to heat changes arising from the passage of electric current through the cell. For aqueous solutions this temperature is in the neighborhood of  $4^\circ \text{C}$ .



## LONGSWORTH'S SCANNING METHOD

The Longworth<sup>17</sup> method uses the optical system shown in Fig. 4. The arrangement is modified, however, by masking the cell image at the photographic plate with a narrow vertical slit and driving the plate horizontally past this slit as the knife edge is progressively raised to the position of the undeviated beam. A contour is thus obtained on the photographic plate which is a graph of the refractive index gradient against the position in the

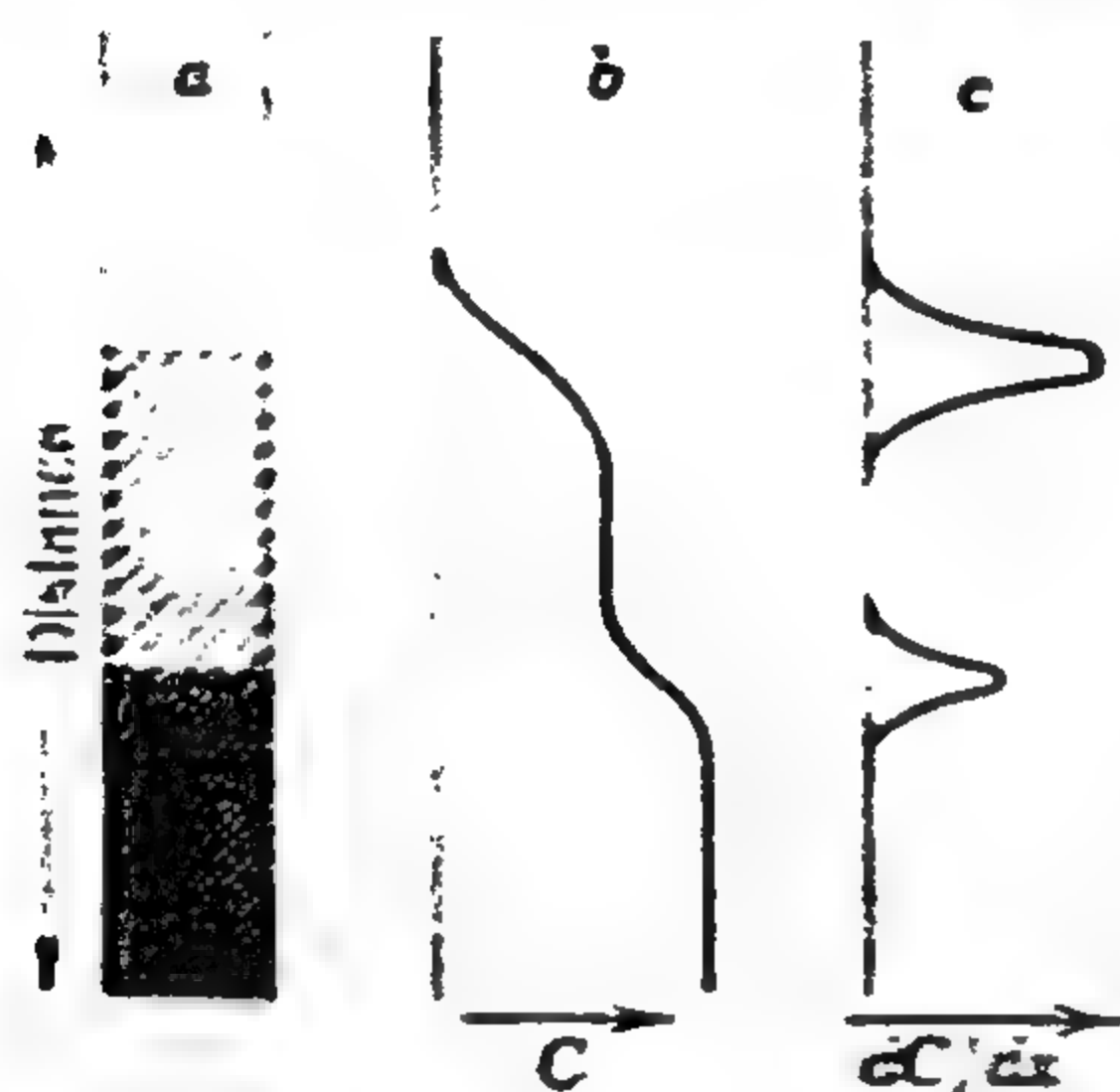


FIG. 7. Concentration of two protein boundaries along electrophoresis cell. : Limit of electrophoresis cell. : Concentration as a function of distance. : Concentration gradients.

electrophoresis cell. The kind of information that can be obtained by the Longworth method is identical with that which may be obtained with the cylindrical lens system.

Shown in Fig. 7 is a plot of the concentration changes at two protein boundaries along the length of the electrophoresis cell compared with the corresponding concentration gradients along the length of the cell.

## MOBILITIES

We have shown above how it is possible to obtain an estimate of the concentrations of the protein components present by measuring the change of the index of refraction gradient along the length of the cell and integrating the area under the curve. Here we wish to outline how the mobilities of the components may be calculated.

The mobility of a particle is evidently given by

$$\mu = \frac{h}{tE} \quad (20)$$

where  $h$  is the distance in centimeters that the protein boundary has moved from its original position in time  $t$  and  $E$  is the potential gradient in volts per centimeter along the length of the electrophoresis cell. Due to the irregularities of the shapes and conductivities through which the electrical current passes, it is not possible simply to divide the difference in potential between the electrodes by the total distance separating them and obtain the potential gradient at any given point in the electrophoresis cell. The amount of current flowing through the electrophoresis cell is given by the relation

$$i = Eq\mu \quad (21)$$

<sup>17</sup> L. G. LONGSWORTH, *J. Am. Chem. Soc.* 61, 529 (1939).

where  $q$  is the cross-sectional area of the electrophoresis cell and  $\kappa$  is the specific conductance of the solution through which the protein molecules are moving. Substituting equation 21 in equation 20 and rearranging, we have

$$u = \frac{hq\kappa}{it} \quad (22)$$

Unfortunately, there is always a lack of symmetry between the ascending and the descending boundaries both in respect to concentration gradient curves as well as the distance moved in a given time. This lack of symmetry in the two limbs of the electrophoretic cell arises from the different ionic environment of the two limbs. The ascending boundary is moving into the buffer solution while the descending boundary is moving into the protein solution. In spite of the fact that the protein solution has been exhaustively dialyzed against the buffer solution before the electrophoretic experiment, the ionic composition and the specific conductance of the two solutions are not identical; the conductance of the protein solution is always less than that of the buffer.

The specific conductance to be used in equation 22 for the calculation of mobilities in the descending limb is clearly that of the original protein solution. The conductance of the solution in which the protein is moving in the ascending limb is, however, neither that of the buffer alone nor of the protein solution alone, and for a completely unambiguous value it would be necessary to measure the conductance just below the ascending boundary. This is a difficult experimental procedure and in the case of complex mixtures of proteins is virtually impossible. Svensson<sup>18</sup> recommends that the velocities of a given component in both limbs of the electrophoresis cell be averaged and that the mean conductivity of the protein solution and of the buffer be used to calculate the mobility.

As pointed out by Longworth and MacInnes,<sup>19</sup> there is an additional correction to be applied to the mobilities which arises from the volume change at the electrodes as a result of electrolysis. This correction, however, amounts to about one per cent of the observed mobilities and except in very exact work is hardly worth applying.

In general, the ascending boundary is sharper and less diffuse than is the descending boundary. As we have noted, the rising boundary moves into a region of higher conductance and lower potential gradient and, accordingly, those particles which by chance move ahead and into a region of lower potential gradient begin to slow down and are overtaken by the rising boundary. Those particles that lag behind the boundary find themselves in a higher potential gradient and, accordingly, speed up. The net effect

<sup>18</sup> H. Svensson, *Ark. Kemi Mineral. Geol.* 22A, No. 10 (1946).

<sup>19</sup> L. G. Longworth and D. A. MacInnes, *J. Am. Chem. Soc.* 62, 705 (1940).



is that the ascending boundary is continuously sharpened. These conditions which lead to sharpening of the boundary do not obtain with the descending boundary, and, accordingly, this boundary becomes diffuse.

It is possible to decrease very considerably the lack of symmetry between the ascending and the descending boundaries by the proper choice of experimental conditions. It has been found that the lower the protein concentration and the higher the ionic strength of the buffer the more closely the ascending and the descending boundaries resemble each other. Longworth<sup>20</sup> has also found that these are the conditions that lead to more accurate estimates of the concentrations and mobilities of mixtures containing two or more proteins. Naturally, if the protein concentration be made too low, the observed refractive index gradient curves of the boundaries are not sufficiently pronounced to obtain an accurate measure of the area under them. If the ionic strength becomes too high, it is not possible to use a high enough potential gradient to separate the protein components.

In addition to the protein boundaries there is always at least one salt boundary or false boundary in each limb of the electrophoresis cell. These boundaries arise as a result of a difference in buffer concentration above and below the original protein boundaries. The false boundary in the descending limb is called the  $\epsilon$ -boundary and that in the ascending side is the  $\delta$ -boundary. These boundaries remain stationary during electrophoresis. Under certain conditions of electrolyte choice it is possible to have additional salt boundaries which move.<sup>21</sup>

### TEST FOR HETEROGENEITY

If a protein solution gives rise to two or more refractive index peaks in addition to the salt boundary upon electrophoresis, obviously the protein solution is a mixture of protein components. This is a rather crude test for heterogeneity. A more critical test is to observe the rate of spreading of the refractive index gradient curve during electrophoresis. That is, owing to small differences in electrophoretic mobility some particles travel faster than others. This gives rise in time to a diffuse boundary with the result that the refractive index gradient peak becomes flatter. However, diffusion alone will also give rise to such spreading and it is, accordingly, necessary to correct for the effect of diffusion before conclusions can be drawn about the electrophoretic heterogeneity of the protein.

Sharp and co-workers<sup>22</sup> first showed that, if the effect of diffusion is

<sup>20</sup> L. G. Longworth, *J. Phys. & Colloid Chem.* **51**, 171 (1947).

<sup>21</sup> H. Svensson, *Ark. Kemi Mineral. Geol.* **17A**, No. 14 (1943); **22A**, No. 10 (1946).

V. P. Dole, *J. Am. Chem. Soc.* **67**, 1119 (1945).

<sup>22</sup> D. G. Sharp, M. H. Hebb, A. R. Taylor, and J. W. Beard, *J. Biol. Chem.* **142**, 217 (1942).



negligible, a measure of the electrophoretic heterogeneity is provided by the standard deviation of the refractive index gradient curve. That is, the refractive index gradient curve has the shape of a probability or Gaussian distribution curve from which it is possible to estimate the standard deviation by statistical methods. The heterogeneity constant is calculated by

$$H = \frac{\Delta\sigma}{\Delta E} \quad (23)$$

where  $\Delta\sigma/\Delta t$  is the rate of change of the standard deviation with time and  $E$  is the potential gradient.

Alberty<sup>23</sup> has considered the more complicated situation where boundary spreading due to diffusion is not negligible but the distribution of mobilities is Gaussian. It is considered that the spreading of the refractive index gradient curve in excess of that due to diffusion results from electrophoretic heterogeneity.

The heterogeneity constant  $h$  may be calculated by the relation

$$\frac{\sigma^2 - \sigma_0^2}{2t} = D + \frac{E^2 h^2 t}{2} \quad (24)$$

where  $\sigma_0$  is the standard deviation of the gradient curve at the moment the electric current is turned on,  $\sigma$  is the standard deviation after electrophoresis

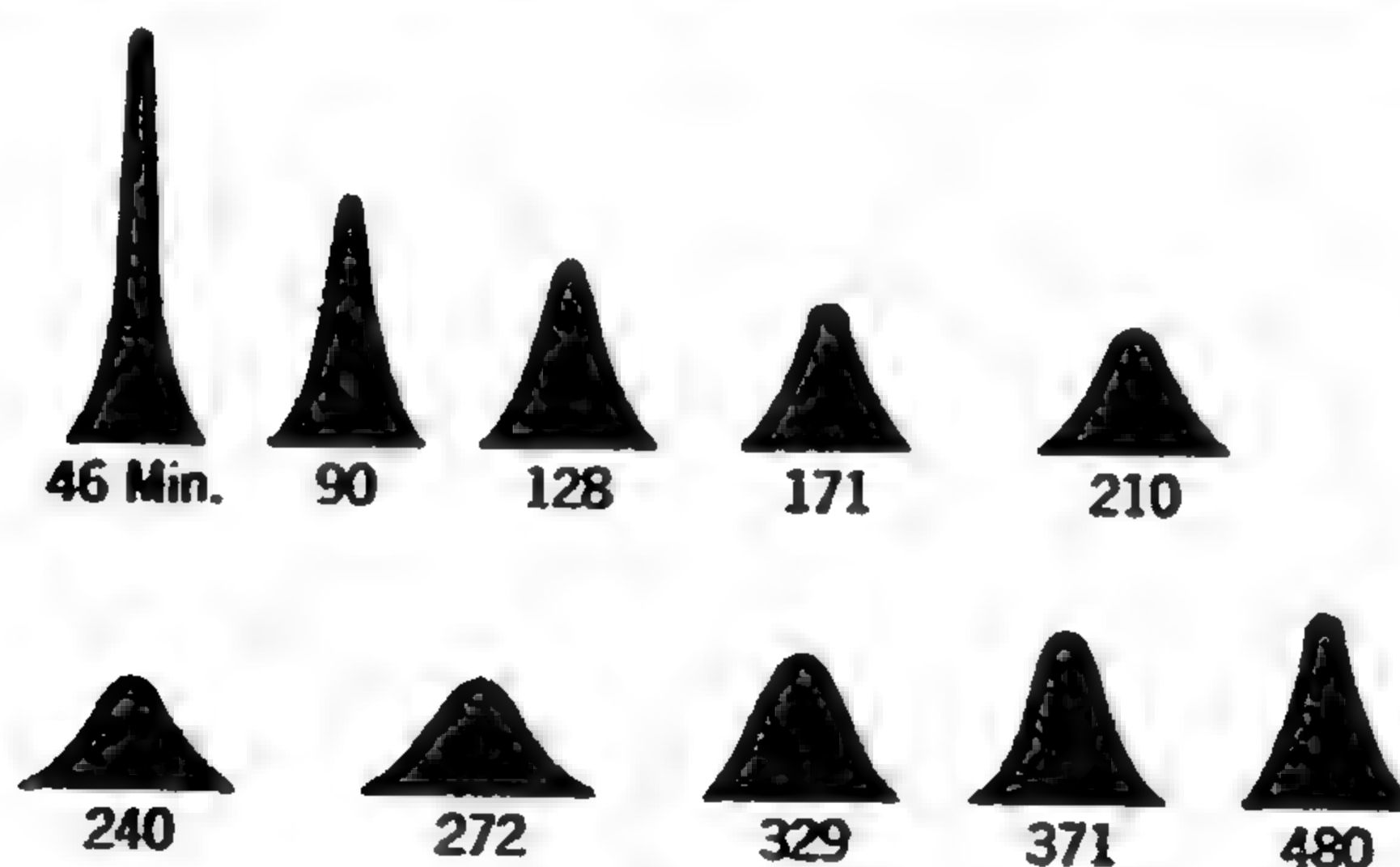


FIG. 8. Refractive index gradient curves for human  $\gamma_2$ -globulin at pH 7.2 during electrophoresis.  $E = 2.40$  volts/cm. Current reversed at 240 min. (Alberty, Anderson, and Williams.)

for  $t$  seconds, and  $D$  is the diffusion constant. Alberty conducts his experiments at the average isoelectric point of the protein. Figure 8 shows the refractive index gradient curves for human  $\gamma_2$ -globulin at pH 7.2, the cur-

<sup>23</sup> R. A. Alberty, *J. Am. Chem. Soc.* 70, 1675 (1948).

R. A. Alberty, E. A. Anderson, and J. W. Williams, *J. Phys. & Colloid Chem.* 52, 217 (1948); 53, 114 (1949).

E. A. Anderson and R. A. Alberty, *J. Phys. & Colloid Chem.* 52, 1345 (1948).

rent being reversed after 240 minutes. Note that the curves sharpen up after reversal of the current: clearly there is electrophoretic heterogeneity present. Apparently, the Alberty test for electrophoretic heterogeneity is a very critical one as even the most highly purified and crystalline proteins show a degree of heterogeneity. The crystalline bovine serum albumin has the smallest constant.

Brown and Cann<sup>24</sup> have treated the more general situation where the mobility does not follow Gaussian probability function and the diffusion is not negligible.

### ELECTROPHORESIS OF COMPLEX MIXTURES

One of the more useful purposes to which electrophoresis has been put has been the analysis of complex mixtures of proteins. The publications

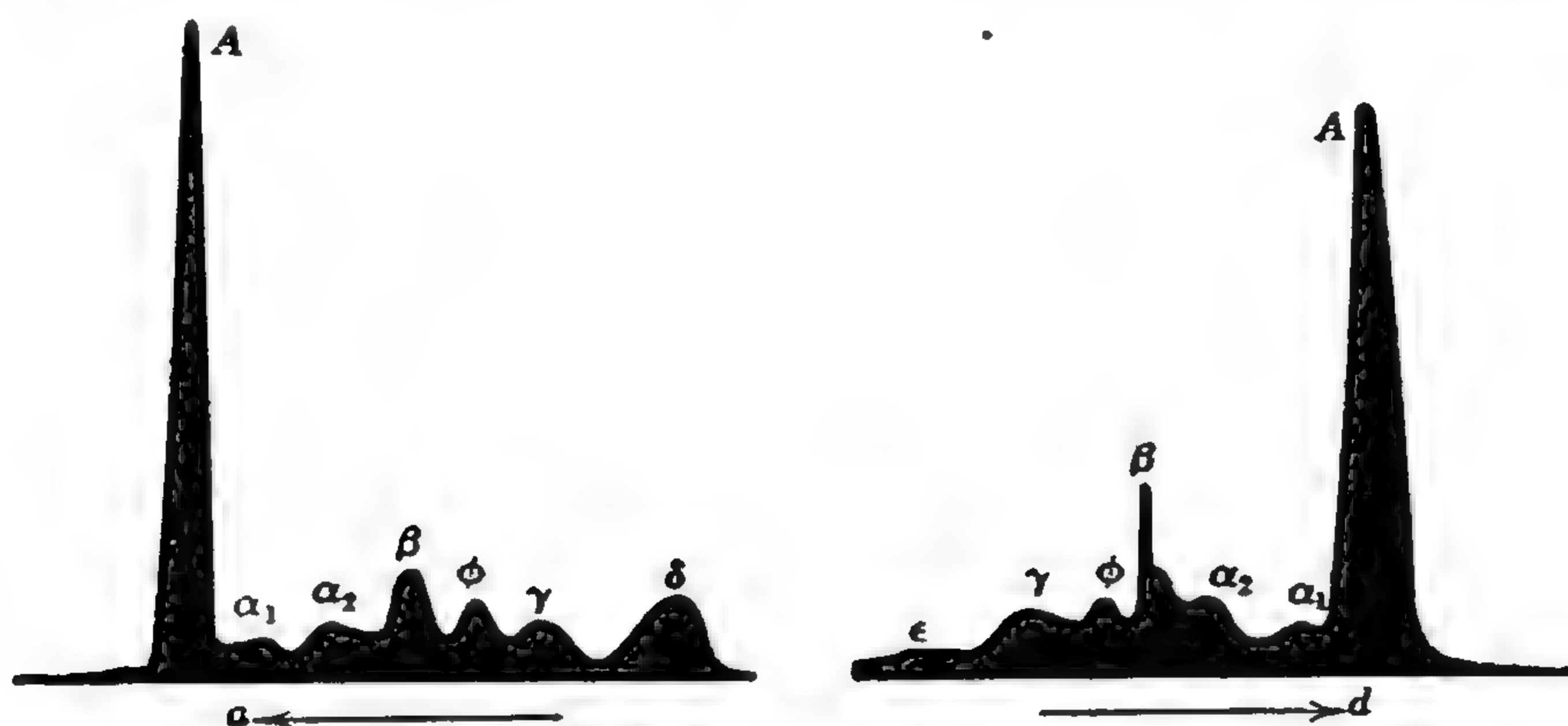


FIG. 9. Electrophoretic pattern of a 1.87 per cent solution of normal human plasma in sodium diethylbarbiturate buffer of pH 8.6 and at 0.1 ionic strength after electrophoresis for 13,020 seconds at 5.21 volts per centimeter (Perlmann and Kaufman). *A* represents albumin,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$  the globulin fractions,  $\phi$  the fibrinogen.  $\delta$  and  $\epsilon$  are the salt boundaries.

dealing with the electrophoresis of blood sera and of blood plasma have been particularly numerous. The literature of electrophoresis of normal blood proteins has been reviewed by Edsall<sup>25</sup> and the electrophoresis of plasma proteins in disease by Stern and Reiner<sup>26</sup> and by Gutman.<sup>27</sup> Shown in Fig. 9 is an electrophoretic pattern of normal human plasma.

Perlmann and Kaufman<sup>28</sup> have investigated the electrophoresis of human blood plasma as a function of the concentration of the total protein and of

<sup>24</sup> R. A. Brown and J. R. Cann, *J. Phys. & Colloid Chem.* **54**, 364 (1950).

<sup>25</sup> J. T. Edsall, *Advances in Protein Chem.* **3**, 392 (1947).

<sup>26</sup> K. G. Stern and M. Reiner, *Fale J. Biol. and Med.* **19**, 67 (1946).

<sup>27</sup> A. B. Gutman, *Advances in Protein Chem.* **4**, 155 (1948).

<sup>28</sup> G. E. Perlmann and D. Kaufman, *J. Am. Chem. Soc.* **67**, 638 (1945).

the ionic strength. They found that the concentrations of the various protein components vary with both the dilution of the plasma and the ionic strength. More satisfactory results appeared to be obtained if the concentration of the plasma proteins was maintained as low and the ionic strength as high as was consistent with good electrophoretic technique.

Other complex mixtures of proteins that have been studied by electrophoresis are the proteins from egg white.<sup>29</sup> The milk proteins have been studied by Warner<sup>30</sup> and by Deutsch<sup>31</sup> and by McMeekin<sup>32</sup> et al.

Muscle proteins have also been studied by electrophoresis.<sup>33, 34</sup>

Two methods are available for distributing the individual areas under the refractive index gradient peaks. These are:<sup>35</sup> an ordinate is drawn from the lowest point between the two adjacent peaks or the pattern is resolved into a series of symmetrical curves.

#### ELECTROPHORESIS AND ACID-BASE TITRATION OF PROTEIN

Abramson<sup>36</sup> pointed out that there should be a relation between the electrophoretic mobility of a protein and the titration curve of a protein, and he formulated the following rule: "In solutions of the same ionic strength, the electric mobility of the same protein at different hydrogen ion activities should be directly proportional to the number of hydrogen (hydroxyl) ions bound." He reasoned that the protein molecule in solution can be pictured as two concentric spheres, the inner sphere being the surface of the protein molecule, and the outer sphere the locus of the center of gravity of the net charge in the diffuse ionic layer.

The relation between the potential and the charge of two concentric spheres is obtained from electrostatic theory (integration of Poisson's equation) and

$$\zeta = \frac{Q}{Dr \left( \frac{r}{d} + 1 \right)} \quad (25)$$

<sup>29</sup> L. G. Longworth, R. K. Cannan, and D. A. MacInnes, *J. Am. Chem. Soc.* **62**, 2580 (1940).

J. A. Bain and H. F. Deutsch, *J. Biol. Chem.* **171**, 531 (1947).

<sup>30</sup> R. C. Warner, *J. Am. Chem. Soc.* **66**, 1725 (1944).

<sup>31</sup> H. F. Deutsch, *J. Biol. Chem.* **169**, 437 (1947).

<sup>32</sup> T. L. McMeekin, B. D. Polis, E. S. Della Monica, and J. H. Custer, *J. Am. Chem. Soc.* **70**, 881 (1948).

<sup>33</sup> W. R. Amberson, T. Erdos, B. Chinn, H. Ludes, *J. Biol. Chem.* **181**, 405 (1949).

<sup>34</sup> P. Crepax, J. Jacob, and J. Seldeslachts, *Biochem. et Biophys. Acta* **4**, 410 (1950).

<sup>35</sup> L. G. Longworth, *Chem. Revs.* **30**, 323 (1942).

<sup>36</sup> H. A. Abramson, *J. Gen. Physiol.* **15**, 575 (1932).



radius of the nucleus of the inner sphere.  $\lambda$  is the distance between the nucleus and the center of the total charge. For spherical particles  $\lambda = r$ .

$$\lambda = \frac{z^{-1}u}{D} \quad (26)$$

where  $u$  is the mobility of the protein molecules in centimeters per volt per centimeter per second and  $\delta$  is a constant which depends on the shape of the particles. We have upon substituting equation 26 into equation 25

$$u = \frac{Q}{3^{-1/r} \left( \frac{r}{\delta} - 1 \right)} \quad (27)$$

The thickness of the double layer  $\delta$  is identified with  $1/\kappa$  of the Debye-Hückel theory (see Chapter 4), and as such its value depends only on the ionic strength of the solution. The thickness of the double layer can thus be maintained constant by means of a series of buffers of different pH but of the same ionic strength, and the mobility of the particles under these isoelectric conditions should be directly proportional to the net charge. The net charge on a protein molecule, as a first approximation is equal to the sum of the hydrogen ions bound or to the sum of the hydrogen ions removed from the protein. As we have seen in Chapter 7, this information can be obtained from an acid-base titration curve of the protein. Atkinson compared the charge calculated by equation 27 with the charge derived from the titration curve and found excellent proportionality. This proportionality was confirmed by Lingsworth,<sup>17</sup> who was able to demonstrate a direct relation between the mobility and the titration curve of egg albumin from pH 3 to 12. Cannan, Palmer, and Klorick<sup>18</sup> have also succeeded in showing a satisfactory relation between these two factors for  $\beta$ -lactoglobulin from cow's milk over a wide range of pH values.

Lingsworth and Jacobsen<sup>19</sup> have studied the binding and release of ions by  $\beta$ -lactoglobulin from milk and by bovine serum albumin by means of electrophoresis. In order to express the electrophoretic mobilities in terms of the charge on the proteins, they compared the mobility of these proteins with the equivalents of protons bound or released from the proteins (Fig. 17 was taken from this publication). The slope of the line  $\Delta u/\Delta i$  for  $\beta$ -lactoglobulin is  $3.2 \times 10^{-6}$ , for bovine serum albumin it is  $2.0 \times 10^{-6}$ .

<sup>17</sup> L. G. Lingsworth, *Ann. N. Y. Acad. Sci.* **41**, 267 (1941).

<sup>18</sup> A. S. Cannan, A. H. Palmer, and A. Klorick, *J. Biol. Chem.* **142**, 535 (1942).

<sup>19</sup> L. G. Lingsworth and C. F. Jacobsen, *J. Phys. & Colloid Chem.* **53**, 126 (1949).

If the value of  $h$  be identified with the net charge on the protein, it is possible to calculate the number of ions adsorbed by the protein from the influence of the ions on the mobility at constant ionic strength.

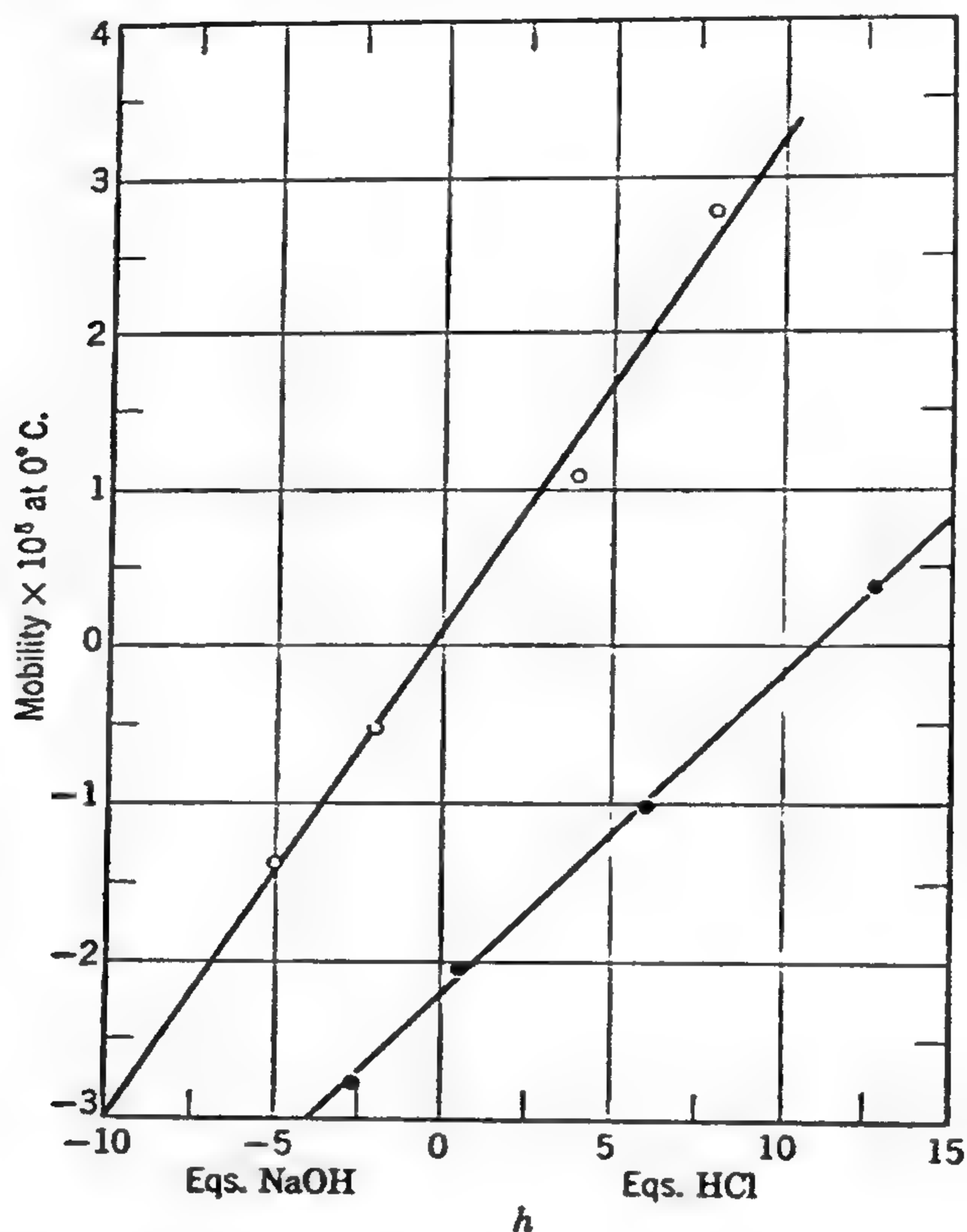


FIG. 10. Plots for the proportionality factor between mobility and titration increment. O,  $\beta$ -lactoglobulin; ●, bovine serum albumin. (Longsworth and Jacobsen.)

### PROTEIN ION INTERACTION

As we noted in Chapter 7, a protein molecule such as that of egg albumin has a multitude of groups capable of binding and releasing protons and at any given  $pH$  such a protein exists in solution in a great many ionic forms. Over a time average, however, there will be associated with each protein molecule a definite number of protons which can be estimated from the titration curve of the protein. These various ionic forms of the protein exist in equilibrium with each other. If the rate of interconversion is high in respect to the separation produced by electrophoresis, this family of ions will travel in the electric field as a single component the mobility of which is

known as the constituent mobility.<sup>40</sup> The constituent mobility is given by the relation

$$\bar{u} = a_1 u_1 + a_2 u_2 + \cdots a_n u_n \quad (28)$$

where  $a_1$  is the fraction of the total number of protein ions with a mobility  $u_1$ ; it is also the fraction of the time the molecules of the constituent exist in the ionic form with mobility  $u_1$ . The meaning of  $a_2, a_3 \cdots a_n$  is similar but refers to the particular ionic form with mobilities  $u_2-u_n$ , respectively.

There exists a constituent mobility not only in regard to proton binding but also in respect to any ion constituent that is reversibly bound. The study of the interaction of proteins with ions by means of electrophoresis has great potential interest because the technique may be applied to the study of the interactions of proteins with other proteins, as in the antigen-antibody reaction as well as reactions involving proteins and nucleic acid in addition to many other types of reactions; these types of interactions are difficult to study by any other method.

Longsworth and MacInnes<sup>41</sup> investigated the interaction of egg albumin with yeast nucleic acid by means of electrophoresis. At pH 5.34 both egg albumin and yeast nucleic acid carry appreciable negative charge, and the electrophoretic pattern for the mixture at this pH was essentially the pattern for the two components obtained separately, thus indicating no interaction. However, at a low pH in the neighborhood of 4.6 there was evidence of marked interaction. Two boundaries were observed in each limb of the electrophoresis cell. The mobility of the slower boundary in the descending limb corresponded to that of egg albumin, whereas the mobility calculated for the faster-ascending peak corresponded to that of the nucleic acid. The mobilities of the other two boundaries did not correspond to the mobilities of either the protein or the nucleic acid but were the constituent mobilities resulting from protein-nucleic acid interaction.

Two notable papers have appeared by Smith and Briggs<sup>42</sup> and by Alberty and Marvin<sup>43</sup> dealing with the theoretical and further experimental aspects of ion interaction with proteins as determined by electrophoresis. These papers are too complex to be dealt with in the space at our disposal except to note that they have succeeded in formulating conditions that permit the calculation of the quantitative extent of ion interaction from electrophoresis.

#### FRACTIONATION BY ELECTROPHORESIS

Under favorable conditions it is possible to fractionate proteins in the ordinary electrophoresis cell. Thus after a time there will exist in the

<sup>40</sup> A. Tiselius, *Nova Acta Regiae Soc. Sci. Upsaliensis* 4, 7, No. 4 (1930).

<sup>41</sup> L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.* 25, 507 (1941).

<sup>42</sup> R. F. Smith and D. R. Briggs, *J. Phys. & Colloid Chem.* 54, 33 (1950).

<sup>43</sup> R. A. Alberty and H. H. Marvin, Jr., *J. Phys. & Colloid Chem.* 54, 48 (1950).



descending limb some of the slowest-moving component which is free of other proteins, and correspondingly in the ascending limb there will be a portion of liquid that will contain a pure solution of the fastest-moving component. The amount of material that can be separated in such a fashion is, however, quite limited, and the components with intermediate mobilities require protracted and involved procedures.

The various designs of apparatus for preparative electrophoresis have been reviewed by Svensson.<sup>44</sup> It would appear that the apparatus developed by Kirkwood et al.<sup>45, 46, 47</sup> using essentially the principle of electrodecantation or electrostratification originally described by Adolf and Pauli<sup>48</sup> appears to be the most promising.

The apparatus consists of two reservoirs connected by a vertical channel, of width sufficiently small to ensure laminar flow, and contains a solution of protein to be fractionated. Upon application of a horizontal electric field, differential transport of the mobile components across the channel takes place, producing a horizontal density gradient depending on the composition gradients. Under the action of gravity, the density gradient induces convective circulation in the channel with a velocity distribution. The result of the superposition of the horizontal electrophoretic transport and vertical convective transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to the slow components and of the bottom reservoir with respect to the fast components.

In order to avoid contamination of the solution by electrolysis products, the walls of the vertical channel are constructed of semipermeable membranes, separated from the electrodes by buffer solution. The electric field across the channel is maintained by the electric current carried by the ions of the buffer electrolyte, to which the membranes are permeable. The exterior buffer solution is replenished by a circulation system at a rate sufficient to prevent electrolysis products from reaching the membranes. Good fractionation of the blood serum proteins is reported.

#### SUMMARY OF ELECTROKINETICS

We have detailed the situation in respect to electrophoresis because this technique has assumed great importance for biochemistry. Streaming potential and electroosmosis, however, are not without interest. There is

<sup>44</sup> H. Svensson, *Advances in Protein Chem.* 4, 251 (1948).

<sup>45</sup> J. G. Kirkwood, *J. Chem. Phys.* 9, 878 (1941).

<sup>46</sup> J. R. Cann, J. G. Kirkwood, R. A. Brown, and O. J. Plescia, *J. Am. Chem. Soc.* 71, 1603 (1949).

<sup>47</sup> J. R. Cann, R. A. Brown, and J. G. Kirkwood, *J. Am. Chem. Soc.* 71, 1609 (1949).

<sup>48</sup> M. Adolf and W. Pauli, *Biochem. Z.* 152, 360 (1924).

in living tissue a constant ebb and flow of fluids, and every such motion must result in a streaming potential.

It is possible to derive equations relating the  $\zeta$ -potential to the streaming potential and to electroosmotic flow; the mathematical procedure is of the same kind as that used by Smoluchowski in the derivation of the equation for the electrophoretic mobility of particles. The relation between the  $\zeta$ -potential and the rate of electroosmotic flow through a capillary is

$$\zeta = \frac{4\pi\kappa\eta V}{Di} \quad (29)$$

where  $V$  is the cubic centimeters flowing per second,  $i$  is the current,  $\kappa$  is the specific conductance in the capillary,  $D$  is the dielectric constant, and  $\eta$  is the viscosity in poise. All electrical quantities in equation 29 are to be expressed in electrostatic units. One electrostatic volt is equal to 299.8 ordinary volts, 1 e.s.u. charge is equal to  $3.33 \times 10^{-10}$  coulomb, and 1 e.s.u. resistance unit is equal to  $8.988 \times 10^{11}$  ohm centimeters. These factors being substituted along with the numerical value of  $\pi$ , equation 29 becomes

$$\zeta = 1.1295 \times 10^6 \frac{\kappa V \eta}{iD} \quad (30)$$

where  $i$  is to be expressed in milliamperes,  $\kappa$  in mhos, and  $V$  in cubic centimeters per second.  $\zeta$  will be calculated in ordinary millivolts. A convenient arrangement of an electroosmotic apparatus has been used by Fairbrother and Balkin.<sup>49</sup> The rate of migration of an air bubble along a calibrated capillary serves as a measure of the rate of flow of the liquid through a membrane inclosed between two electrodes. The capillary and membrane constitute a closed system, and any displacement of the liquid in the membrane has to produce a motion of the air bubble in the connected capillary. Non-polarizable electrodes are necessary.

The corresponding equation for streaming potential is

$$\zeta = \frac{4\pi\eta\kappa H}{DP} = 8.513 \times 10^8 \frac{\eta\kappa H}{DP} \quad (31)$$

The second term in equation 31 involves the use of electrostatic units; the third term includes the numerical factors.  $H$  is the streaming potential measured across the ends of the capillary,  $\eta$  is the coefficient of viscosity of the liquid in poise,  $P$  is the pressure forcing the liquid through the capillary, and  $\kappa$  is again the specific conductance of the liquid in the capillary. The third term in equation 31, which includes the numerical factors, expresses

\* F. Fairbrother and M. Balkin, *J. Chem. Soc.* 1931, 389.

$H$  and  $\zeta$  in ordinary millivolts and  $P$  in centimeters of mercury pressure (density of mercury at 25° C. is 13.53). The streaming potential  $H$  must be measured with a high-resistance apparatus such as a quadrant electrometer or a vacuum-tube potentiometer; otherwise the charge built up at the two ends of the capillary will discharge through the measuring apparatus.

It was first pointed out by Briggs<sup>50</sup> that the specific conductance to be used in equations 30 and 31 must be the conductance in the capillaries through which the liquid is passing. Owing to the accumulation of ions in the neighborhood of the solid wall the specific conductance of a liquid in a capillary is increased above that in bulk. Briggs measured the resistance of the capillary filled with liquid being investigated and then replaced the liquid with a fairly concentrated KCl solution and thus determined the cell constant of the capillary. The specific conductance of the original liquid in the capillary was then calculated in the usual manner. The increase of the conductance above that found in bulk is called the specific surface conductance, and under certain conditions this surface conductance can amount to a large fraction of the total conductance. For example, the specific conductance of water in a certain packed cellulose membrane was found to be  $31.3 \times 10^{-6}$  mho, whereas the specific conductance of this same water in bulk was only  $4.8 \times 10^{-6}$  mho.

The surface conductance is due to at least two factors;<sup>51</sup> (1) the movement of the charge at the interface which is a true electroosmosis and (2) the accumulation of neutral salts in the neighborhood of the interface in excess of that in the bulk of the liquid. The electroosmotic part of the surface conductance was formulated by Smoluchowski.<sup>52</sup> It is possible that the electroosmotic factor is important at low salt concentration but with increasing salt concentration the part of the surface conductance due to the accumulation of neutral salts at the solid surface becomes increasingly larger and finally outweighs the electroosmotic part of the surface conductance.

Jones and Wood<sup>53</sup> as well as Rutgers and de Smet<sup>54</sup> have made careful measurements of the streaming potential through glass capillaries. It is notable that both groups of workers report a linear relation between the calculated  $\zeta$ -potential and the logarithm of the salt concentration. For a uni-univalent salt the slope of this line is about 25 millivolts per unit log

<sup>50</sup> D. R. Briggs, *J. Phys. Chem.* 32, 641 (1928).

<sup>51</sup> J. J. Bikermann, *Kolloid-Z.* 72, 100 (1935); *Trans. Faraday Soc.* 36, 154 (1940).

<sup>52</sup> M. v. Smoluchowski, *Anz. Akad. Wiss. Krakau (A)* 1912, 635.

<sup>53</sup> G. Jones and L. A. Wood, *J. Chem. Phys.* 13, 106 (1945).

L. A. Wood, *J. Am. Chem.* 68, 437 (1946).

<sup>54</sup> A. J. Rutgers and M. de Smet, *Trans. Faraday Soc.* 43, 102 (1947).



change in concentration. Thus the maximum in the  $\zeta$ -potential so often reported at lower salt concentrations in earlier publications apparently is incorrect.

The utility of streaming potential and electroosmotic experiments would be greatly extended if such measurements could be made on membranes. In spite of a large amount of work along these lines the situation remains ambiguous. Thus White, Urban, and Krick<sup>55</sup> reported that as the radius of glass capillaries is decreased the calculated  $\zeta$ -potential decreases. Bull and Gortner<sup>56</sup> measured the streaming potential of  $2 \times 10^{-4} M$  sodium chloride in a series of powdered quartz diaphragms. The quartz had been fractionated according to particle size. Below a certain critical particle size the  $\zeta$ -potential decreased sharply with decreasing particle size. Such results serve to cast considerable doubt on the validity of streaming potential and electroosmotic experiments made on diaphragms and membranes. There has been much speculation on electrokinetics of membranes without, however, arriving at any useful conclusions.<sup>57, 58, 59</sup>

#### COMPARISON OF ELECTROKINETIC METHODS

We can show that the equations for electroosmosis and for streaming potentials obey certain simple requirements. Velisek and Vasicek<sup>60</sup> have found that, in accord with equation 29, when the volume of liquid flowing is plotted against the current, a straight line is obtained. They used membranes made of porous porcelain. Bull<sup>61</sup> was able to show that the plot of the streaming potential against the hydrostatic pressure also yields a straight line as demanded by equation 31. He used Pyrex-glass capillaries.

It has been shown that the methods of electroosmosis, of streaming potential, and of electrophoresis are consistent in that they yield the same calculated  $\zeta$ -potential for identical surfaces in identical electrolyte solutions. The microelectrophoretic method provides a way for determining not only the electrophoretic mobility but also the electroosmotic mobility. Abramson<sup>62</sup> has pointed out that in a rectangular microelectrophoretic cell

$$U_e = 2(U_{1/2} - U_e) \quad (32)$$

where  $U_{1/2}$  is the observed mobility at the midpoint of the cell,  $U_e$  is the electrophoretic mobility,  $U_e$  is the electroosmotic velocity of flow of the

<sup>55</sup> H. L. White, F. Urban, and E. T. Krick, *J. Phys. Chem.* **36**, 120 (1932).

<sup>56</sup> H. B. Bull and R. A. Gortner, *J. Phys. Chem.* **36**, 111 (1932).

<sup>57</sup> J. J. Bikermann, *J. Phys. Chem.* **46**, 724 (1942).

<sup>58</sup> H. B. Bull and L. S. Moyer, *J. Phys. Chem.* **40**, 9 (1936).

<sup>59</sup> S. Komagata, *Researches Electrochem. Lab. Tokyo*, No. 362 (1934).

<sup>60</sup> J. Velisek and A. Vasicek, *Z. physik. Chem.* **A71**, 281 (1934).

<sup>61</sup> H. B. Bull, *Kolloid-Z.* **66**, 20 (1934).

<sup>62</sup> H. A. Abramson, *Colloid Symposium Monograph* **8**, 289 (1930).

water past the cell wall. Moyer and Abramson<sup>13</sup> coated quartz particles and the inside surfaces of the electrophoretic cell with protein. The particles and cell walls, accordingly, had identical surfaces. Under these conditions the electroosmotic mobility of the water past the cell surface was found to be equal to the electrophoretic mobility of the coated quartz particles. This shows that electrophoresis and electroosmosis are consistent with each other. Incidentally, if the surfaces of the particles and of the inside surfaces of the electrophoretic cell are identical,  $U_1$  equals  $U_2$  and, from equation 32,  $U_c$  must equal  $\frac{2}{3}U_1$ . This allows the electrophoretic mobility to be calculated from the particle mobility at one-half the depth of the electrophoretic cell. Experimentally, the half thickness is the most convenient place to make measurements. Bull<sup>14</sup> performed the same type of experiments on protein-covered surfaces as did Moyer and Abramson, and he included in his studies streaming potentials through glass capillaries covered with protein. He was able to show that streaming potential, electrophoresis, and electroosmosis are all consistent with one another.

Since it has been shown experimentally that the methods of electrophoresis, electroosmosis, and streaming potential are equivalent to one another, it would appear that the best way of expressing electrokinetic data is by means of a function common to all three methods. Mobility in  $\mu$  per second per volt per centimeter is widely used to express electrophoretic data. It is proposed to use this term also for electroosmotic and streaming-potential data. This is quite a reasonable method of expression for electroosmosis and would have a definite physical meaning. It would have no direct physical meaning for streaming potential but would bring this technique into line with the other methods without the use of the  $\zeta$ -potential.

The numerical equivalence of these expressions using ordinary electrical units and expressing  $P$  in centimeters of mercury is

$$7.54 \times 10^3 \frac{\kappa H}{P} = 10.00 \frac{\kappa V}{i} = u \quad (33)$$

where  $H$  is expressed in ordinary volts,  $i$  in amperes,  $V$  in cubic centimeters per second,  $\kappa$  in mhos, and  $u$  in  $\mu$  per second per volt per centimeter.  $\kappa H/P$  is for streaming potential;  $\kappa V/i$  is for electroosmosis; and  $u$  is for electrophoresis.

#### STRUCTURE OF THE ELECTRICAL DOUBLE LAYER

Helmholtz, who did the first important theoretical work on electrokinetics, considered that an electrical double layer of fixed thickness existed at the surface. This layer was assumed to be of the order of one

<sup>13</sup> L. S. Moyer and H. A. Abramson, *J. Gen. Physiol.* 19, 727 (1936).

<sup>14</sup> H. B. Bull, *J. Phys. Chem.* 39, 577 (1935).



molecule in thickness and could be represented by two parallel flat plates. He further considered the dielectric constant to have the value of unity. Gouy<sup>65</sup> later pointed out that the double layer at the interface could not be rigid, compact, and of fixed thickness, but must be diffuse. He pictured the situation as follows: At very low temperatures ions are rigidly adsorbed on the surface, thus forming a true Helmholtz layer, but as the temperature is raised, say to room temperature, a large fraction of the adsorbed ions "vaporize" from the surface. However, these ions are not able to escape entirely from the influence of the charge on the wall. This ionic atmosphere in the immediate neighborhood of the surface is fairly dense, and at greater distances from the surface the density diminishes until the net charge density is zero. Not all the ions in the diffuse double layer are of opposite sign from those on the wall, but the ions of opposite sign do predominate. In view of the fact that the diffuse layer extends some distance into the water phase, it is necessary to consider the dielectric constant; this constant is assigned the value it has in pure water. Such was Gouy's picture. On the basis of this concept of the double layer, he was able to derive equations relating the net charge density to the distance between the center of gravity of the net charge in the diffuse layer and the center of gravity of the charges fixed on the solid surface and to the potential of the double layer. He also formulated an equation for the thickness of the double layer. Thus in 1910 an adequate mathematical theory of the double layer was available, but it was not until several years after the advent of the Debye-Hückel theory of strong electrolytes that the importance of these ideas for electrokinetic phenomena was realized.<sup>66</sup>

The same type of mathematical approach was used by Gouy as was later employed by Debye and Hückel (see Chapter 4). By means of the Boltzmann principle, the charge density of the diffuse layer was calculated. This was substituted in Poisson's equation and the equation integrated to yield a relation between charge density and the  $\zeta$ -potential. The electrostatic charge per square centimeter of surface is given by

$$\sigma = \sqrt{\frac{DRT}{2000\pi}} \sqrt{C_c(e^{-z_c\epsilon\zeta/kT} - 1) + C_a(e^{z_a\epsilon\zeta/kT} - 1)} \quad (34)$$

where the subscript  $c$  applies to cations and the subscript  $a$  to anions. In water at 25° C.,

$$\sigma = 1.765 \times 10^4 \sqrt{C_c(e^{-0.039z_c\zeta} - 1) + C_a(e^{0.039z_a\zeta} - 1)} \quad (35)$$

<sup>65</sup> L. Gouy, *J. phys.* **9**, 457 (1910).

<sup>66</sup> E. F. Burton, *Colloid Symposium Monograph* **4**, 132 (1926).

H. A. Abramson, *Electrokinetic Phenomena*, The Chemical Catalog Co., Inc., New York 1934.



where  $\zeta$  is expressed in ordinary millivolts and  $\sigma$  is obtained as the number of electrostatic charges per square centimeter. For uni-univalent salts in water at 25° C.,

$$\sigma = 3.53 \times 10^4 \sqrt{C} \sinh 0.039\zeta \tag{36}$$

where  $\sinh$  is the hyperbolic sine. Tables of hyperbolic functions are available in most physical and chemical handbooks.

The thickness of the double layer is considered to be the distance between the center of gravity of the fixed layer on the solid surface and that of the diffuse layer. It is equal to the reciprocal of  $\kappa$  of the Debye-Hückel theory, and

$$d = \frac{1}{\kappa} = \sqrt{\frac{1000DRT}{8\pi\epsilon^2N^2\mu}} \tag{37}$$

where  $d$  is the thickness of the double layer and  $\mu$  is the ionic strength. In water at 25° C.

$$d = \frac{3.05 \times 10^{-8}}{\sqrt{\mu}} \text{ centimeter} \tag{38}$$

Note that the thickness of the double layer is independent of the potential and charge of the layer and at any given temperature depends only on the ionic strength. The value of the constants and variables to be used in equations 34 and 37 are given in Table 1.

TABLE 1  
CONSTANTS AND VARIABLES TO BE USED IN EQUATIONS 34 AND 37

Function	Name	Value
$\epsilon$	Elementary charge	$4.80 \times 10^{-10}$ e.s.u.
$k$	Boltzmann constant	$1.38 \times 10^{-16}$ erg per degree
$N$	Avogadro's constant	$6.023 \times 10^{23}$
$R(kN)$	Molar gas constant	$8.315 \times 10^7$ ergs per degree
$D$	Dielectric constant	78.54 water at 25° C.
$\pi$		3.1416
$T$	Absolute temperature	
$z_c$	Valence of cation	
$z_a$	Valence of anion	
$C_c$	Moles cations per liter	
$C_a$	Moles anions per liter	

Table 2 shows the variation of the thickness of the double layer with various concentrations of different types of electrolytes.

TABLE 2

THICKNESS OF THE ELECTRICAL DOUBLE LAYER IN THE PRESENCE OF  
ELECTROLYTES

Concentration Moles per Liter	Thickness of Double Layer in Å		
	Uni-Univalent	Uni-Divalent	Di-Divalent
$10^{-5}$	965	556	484
$10^{-3}$	96.5	55.6	48.4
$10^{-1}$	9.65	5.56	4.84

## ELECTROSTATIC FORCES

It has long been considered that the  $\zeta$ -potential is one of the principal factors responsible for the stability of hydrophobic colloidal suspension such as is represented by a gold sol. That is, in order to bring two such gold particles together, the electrostatic repulsive forces arising from the  $\zeta$ -potential must be overcome. Such electrostatic forces have also been evoked to explain the formation of certain types of gels, specifically, the formation of thixotropic gels whose gel structure can be disturbed by mechanical agitation but whose structure re-forms when the agitation is stopped. Certain iron oxide sols form, upon standing, what are known as Schiller planes which are made up of parallel layers of plate-like iron oxide particles having uniform spacings which may be as great as 8000 Å. The maintenance of these uniform distances of separation have been attributed to the action of electrostatic repulsive forces.<sup>67</sup>

Hamaker<sup>68</sup> considered that the potential energy between two colloidal particles consisted of two parts, a negative contribution due to van der Waals' attraction and a contribution due to the interaction of the charges of the electrical double layers around the particles. This problem has been considered by others.<sup>69, 70, 71, 72, 73</sup> The treatment of this problem by Verwey and Overbeek<sup>74</sup> appears more complete than any of the others. They consider that the van der Waals forces are attractive, whereas the forces resulting from double-layer interaction (for particles bearing like sign of charge) are always repulsive.

It is less complicated to consider the interaction between two parallel

<sup>67</sup> P. Bergmann, P. Löw-Beer, and H. Zocher, *Z. Physik. Chem.* A181, 301 (1938).

<sup>68</sup> H. C. Hamaker, *Chem. Weekblad* 35, 47 (1938).

<sup>69</sup> B. Derjaguin, *Trans. Faraday Soc.* 36, 203 (1940).

<sup>70</sup> S. Levine, *Proc. Roy. Soc. (London)* A170, 145, 165 (1939).

<sup>71</sup> S. Levine and G. P. Dube, *Trans. Faraday Soc.* 35, 1125 (1939); 36, 215 (1940).

<sup>72</sup> Corkill and Rosenhead, *Proc. Roy. Soc. (London)* A172, 410 (1939).

<sup>73</sup> I. Langmuir, *J. Chem. Phys.* 6, 873 (1938).

<sup>74</sup> E. J. W. Verwey and J. T. G. Overbeek, *Theory of the Stability of Lyophobic Colloids*, Amsterdam, Elsevier Publishing Co., 1948; *Trans. Faraday Soc.* 42B, 117 (1946).

charged plates rather than between two colloidal particles. Also it appears easier to calculate the work required to bring two such plates towards each other rather than to consider the forces involved. A choice has to be made as to whether the surface potential of the plates is to be considered constant or the charge is to be held constant. Verwey and Overbeek assume the surface potential to be constant. According to this method of approach, the free energy of the double-layer system is found as the total amount of work associated with the gradual discharge of all ions of the system, including the ions responsible for the surface charge. Carrying out such calculations, they find for slight interaction of the two double layers of the plates that the free energy per square centimeter required to bring two plates from infinity to distance  $d$  apart is

$$\Delta F = \frac{32nkT}{\kappa} \gamma^2 e^{-2\kappa d} \quad (39)$$

where  $n$  is the number of ions per cubic centimeter,  $\kappa$  is the reciprocal of the thickness of the double layer,  $k$  is Boltzmann's constant,  $T$  is the absolute temperature, and  $\gamma$  is equal to  $(e^{y/2} - 1)/(e^{y/2} + 1)$ , where  $y$  is equal to  $ze\zeta/kT$ , in which  $z$  is the valence of the ion,  $e$  is the elementary charge, and  $\zeta$  is the potential of the double layer expressed in electrostatic volts. For a uni-univalent electrolyte at 25° C. the equation reduces to

$$\Delta F = \frac{24.2c}{\sqrt{c}} \gamma^2 e^{-2\kappa d} \quad (40)$$

where  $c$  is now expressed in moles per liter. It can be seen that, for large values of  $y$ ,  $\gamma^2$  approaches unity and, in general, the potential energy between two plates is not particularly sensitive to variation in  $\zeta$ .

A consideration of the electrostatic forces of repulsion rather than the free energy change yields

$$\text{Repulsive force} = 64n \left( \tanh \frac{e\zeta}{kT} \right)^2 e^{-2\kappa d} \quad (41)$$

The combination of the electrostatic repulsive forces due to double-layer interaction with the van der Waals attractive forces leads to a series of complicated force-distance curves. The individual shapes of these curves are functions of the electrolyte concentration, the  $\zeta$ -potential, and the nature of the van der Waals interaction. As the distance between the plates is decreased, the net repulsive force increases in magnitude until at quite short distances the van der Waals attractive forces exceed the electrostatic repulsion and cohesion of the plates results.



Apparently, the calculation of the van der Waals attractive forces is on a much less sound basis than is the treatment of the electrostatic repulsive forces. According to Hamaker, the attractive van der Waals potential between two parallel plates one square centimeter in area is

$$V = - \frac{A}{48\pi} \frac{1}{d^2} \quad (42)$$

where  $A$  is a constant whose value is not exactly known but is supposed to lie between  $10^{-12}$  and  $10^{-13}$ .

These calculations of Verwey and Overbeek are certainly most interesting and they are probably, in principle, correct. It is, however, the feeling of the author that, until suitable experiments can be devised to test this theory in a quantitative and unambiguous manner, decision as to its validity should be suspended. Discussion of long-range repulsive forces is continued in Chapter 12.

### PROBLEMS AND QUESTIONS

1. Describe how you would determine the isoelectric point of a protein by means of the microelectrophoretic technique.

2. The specific conductance of a buffered protein solution is  $6.00 \times 10^{-4}$ , the cross-sectional area of the electrophoresis cell is 30 sq. mm., the current is 10 ma., and the protein boundary moves 1.43 cm. in 2 hours. Calculate the electrophoretic mobility of the moving protein boundary in  $\mu/\text{sec}/\text{volt}/\text{cm}$ . *Ans.: 0.036  $\mu/\text{sec}/\text{volt}/\text{cm}$ .*

3. The mobility of a human red cell at  $25^\circ \text{C}$ . in  $M/15$  phosphate buffer at  $p\text{H}$  7.4 is  $1.31 \mu/\text{sec}/\text{volt}/\text{cm}$ . Calculate the zeta potential and the thickness of the electrical double layer. *Ans.:  $\zeta = 16.85 \text{ mv.}$ ; thickness =  $7.6 \times 10^{-8} \text{ cm}$ .*

4. Explain why in the moving-boundary electrophoretic technique the ascending boundary is, in general, sharper than is the descending boundary.

5. What is meant by surface conductance and how does it enter into electrokinetic theory?

## SURFACE ACTIVITY

It is a familiar observation that a sewing needle, if placed carefully on a clean water surface, will float: the water behaves as if it had a skin. The surface of water is really under a considerable tension: the needle does not have sufficient mass to overcome this tension, and as a result it floats.

The physical origin of surface tension may be pictured somewhat as follows: In the body of the liquid the molecules are acted upon in all directions by the attractive forces of other liquid molecules. The molecules in the surface, however, are attracted only downward and sideways. The molecules in the body of the liquid thus have a lower potential energy than those on the surface, and, accordingly, in order to bring a molecule from the interior of the liquid to the surface, work must be done. Any extension of the surface requires the expenditure of energy.

Surface tension can be expressed in one of two ways, which are numerically and dimensionally equivalent, namely, in dynes per centimeter or in ergs per square centimeter, i.e., in force per unit length or in work per unit area. The dimensions of surface tension are  $M l^2$ .

The surface tension of pure liquids is independent of extension: i.e., no matter how greatly the surface is extended, the work required to produce an additional square centimeter of surface is the same as it was initially. Rubber is a bad analogy for the surface tension of pure liquids because, as is well known, the force required to extend rubber is proportional to the extension. Under some conditions, however, liquid surfaces do show elasticity, i.e., the surface tension is proportional to the extension. The surfaces of dilute solutions of surface-active substances as well as surfaces of spread monomolecular films show elasticity. Such surfaces will be considered presently.

The thickness of the surface film of a pure liquid is undoubtedly a function of the nature of the liquid. By such a thickness we mean, of course, the transition distance between the uniform conditions of the gaseous phase to the uniform conditions of the liquid phase. McBain et al.<sup>1</sup> report from

<sup>1</sup> J. W. McBain, R. C. Bacon, and H. D. Bruce, *J. Chem. Phys.* 7, 818 (1939).

optical measurements that the least possible thickness of the water surface is 2 to 3 Å.

### MEASUREMENT OF SURFACE TENSION

Numerous methods are available for the measurement of surface tension. In general, they may be divided into two classes, static and dynamic. Some methods do not belong to either class but are intermediate between the truly static and truly dynamic methods. The static surface tension is the

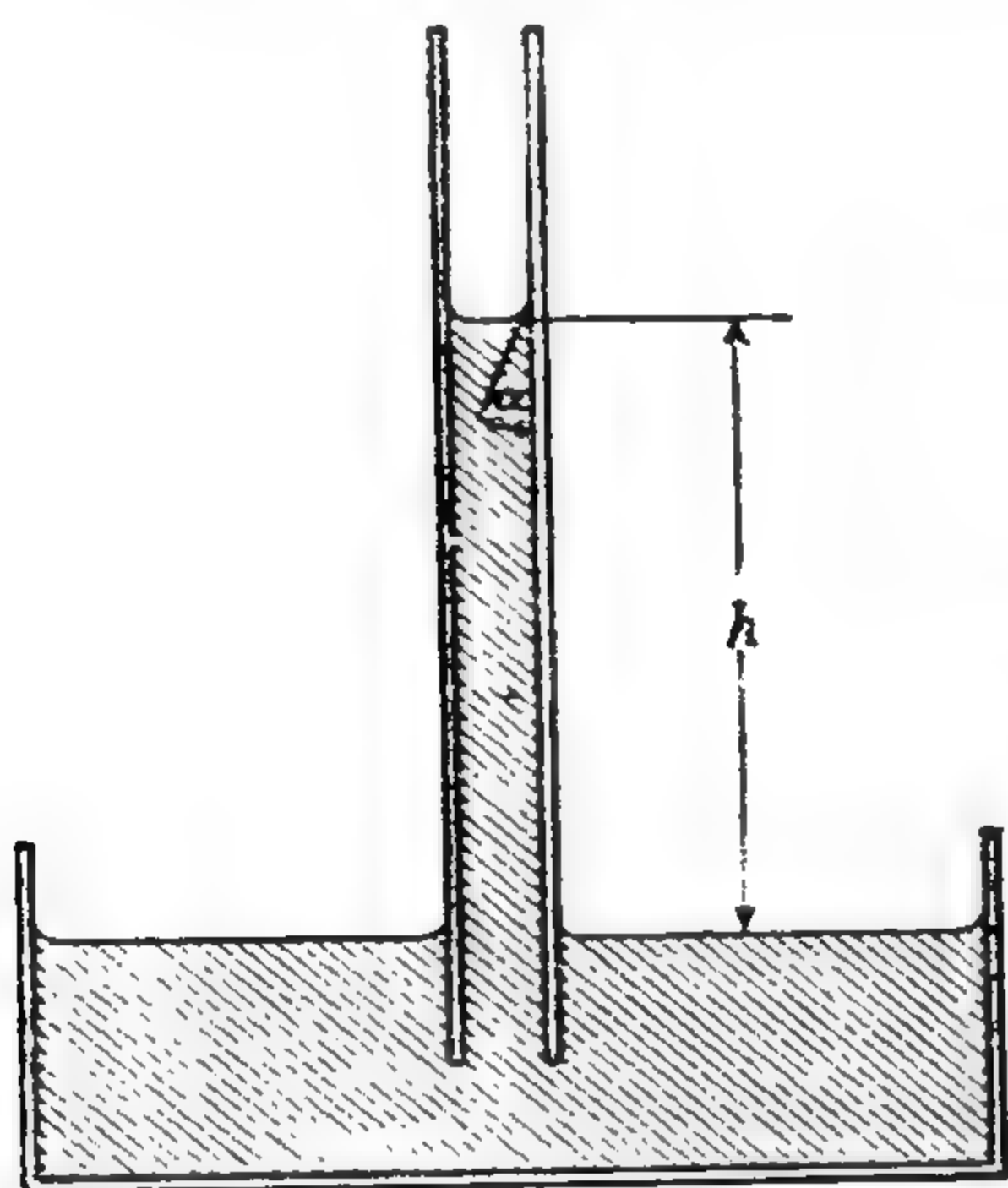


FIG. 1. Rise of a liquid in a capillary.

equilibrium tension; the dynamic tension is the tension of a liquid before the surface film has had time to form. The time required for the attainment of surface equilibrium of pure liquids is exceedingly small, probably of the order of a millionth of a second. The time required for a solution in which the solute molecules are of moderate size to reach equilibrium is also exceedingly short. It is only when one deals with solutions of colloidal materials that the time required for the formation of the surface is appreciable. We shall confine our attention to methods that give or are supposed to give a static surface tension. The most important and useful of these static methods are the

capillary-rise method, the du Noüy ring method, the Wilhelmy balance method, and the drop-weight method.

*The capillary-rise method* is a truly static one. It consists in measuring the height of rise of the liquid in a capillary. Consider a capillary of radius  $r$ , into which ascends a liquid to height  $h$ , above the surface of the liquid. This liquid is to make an angle of contact  $\alpha$  with the walls of the capillary (see Fig. 1). It is evident that the force pulling the liquid up is  $2\pi r\sigma \cos \alpha$ , while the force pulling downward is  $\pi h r^2 g \rho$ , where  $\sigma$  is the surface tension,  $g$  is the acceleration of gravity, and  $\rho$  is the density of the liquid. At equilibrium the upward and downward directed forces must equal each other, and

$$\sigma = \frac{h r g \rho}{2 \cos \alpha} \quad (1)$$

In practice only those liquids are employed that completely wet the surface of the capillary. Under this condition, the angle of contact is zero and  $\cos \alpha$  is unity. The difficulty with the method is that the bore of the



capillary must be exactly uniform—a requirement hard to meet. It is not so important that the inside of the capillary be an exact cylinder: i.e., the cross section of the capillary may be slightly elliptical without seriously affecting the accuracy of the measurement. The capillary-rise method is entirely unsuited for the measurement of the surface tension of protein solutions; the protein surface films tend to stick in the capillary and prevent the solution from reaching its equilibrium height. This method, accordingly, is severely limited in its application to the determination of the surface tension of biological fluids. Critical papers dealing with this method have been published by Richards and Carver,<sup>2</sup> by Harkins and Brown,<sup>3</sup> and by Jones and Frizzell.<sup>4</sup>

Jones and Ray<sup>5</sup> have devised a differential method based on the capillary-rise technique which is capable of extreme accuracy. The apparatus consists of a wide tube and a narrow one in the shape of a U. The liquid is brought to a fixed mark in the narrow tube by the addition of liquid to the larger tube. The main feature is that the difference in levels of the narrow and the wide menisci is found by weighing the total amount of liquid in the apparatus. The level of the liquid in the smaller tube being fixed, it is not necessary to insist upon extreme uniformity of the diameter of the smaller tube. With this apparatus Jones and Ray were able to obtain some interesting and unexpected results on the surface tension of aqueous solutions of inorganic electrolytes. Previous to their work it was thought that inorganic electrolytes, throughout their entire concentration range, raised the surface tension of water.<sup>6</sup> Jones and Ray found characteristic minima in the concentration-surface tension curves at very low electrolyte concentrations. Langmuir<sup>7</sup> has proposed a theory of the Jones-Ray effect in which he suggested that the minima in the surface tensions were only apparent and concluded that they resulted from the electrostatic charges on the surface of the glass capillary. The theoretical aspects of this problem have been further commented on.<sup>8</sup>

*The du Noüy ring method* has the advantage of simplicity, and the complete apparatus is commercially available. The method consists in determining the force required to detach a platinum ring from the surface of a liquid. The simple theory requires that the surface tension shall be the force required to pull the ring out of the surface divided by twice the cir-

<sup>2</sup> T. W. Richards and E. K. Carver, *J. Am. Chem. Soc.* 37, 1656 (1915).

<sup>3</sup> W. D. Harkins and F. E. Brown, *J. Am. Chem. Soc.* 41, 503 (1919).

<sup>4</sup> G. Jones and L. D. Frizzell, *J. Chem. Phys.* 8, 986 (1940).

<sup>5</sup> G. Jones and W. A. Ray, *J. Am. Chem. Soc.* 59, 187 (1937).

<sup>6</sup> L. Onsager and N. N. T. Samaras, *J. Chem. Phys.* 2, 528 (1934).

<sup>7</sup> I. Langmuir, *Science* 88, 430 (1938).

<sup>8</sup> A. S. Coolidge, *J. Am. Chem. Soc.* 71, 2153 (1949).

circumference of the ring. The distance is twice the circumference because two films are involved, an inner and an outer one. Actually, the surface tension as measured by the tensiometer is sensitive to the diameter both of the ring and of the wire. Large errors may be introduced by the use of rings of improper diameter. In spite of the acknowledged errors to which this method is subject, it is surprising how frequently quite accurate results are obtained with the du Noüy tensiometer. Good references to this method are papers by Harkins and Jordan,<sup>9</sup> by Freud and Freud,<sup>10</sup> and by Hauser.<sup>11</sup>

Dole and Swartout<sup>12</sup> have devised a differential method involving the use of two large platinum rings. One ring dips into pure water and the other into the solution whose surface tension is to be measured. The rings are supported from the ends of the beam of an analytical balance. The liquids are simultaneously lowered, and the ring which is detached is noted. Weights are added or subtracted from the balance until a small change of weight will cause one or the other rings to break from the surface. By comparing this weight with the weight found when both pans contain pure water, an accurate value of the relative surface tension of the solution is obtained. Dole and Swartout investigated the surface tension of aqueous solutions of electrolytes and obtained the characteristic minima in the surface tension-concentration relation at low electrolyte concentrations as reported by Jones and Ray.<sup>3</sup>

*The Wilhelmy balance method* was devised by Wilhelmy<sup>13</sup> a number of years ago and revived by Dervichian.<sup>14</sup> The apparatus consists simply of a thin strip of glass dipping into the liquid under investigation and suspended from an arm of an analytical balance (a chainomatic balance is most convenient). The weight of the dry slide in air and its weight while dipping into the liquid are determined. After the buoyancy correction of the liquid displaced by the slide is added, and the weight of the slide in air subtracted from the weight when dipping in the liquid, the surface tension can be directly calculated. The net pull of the surface on the slide in grams is multiplied by the acceleration of gravity and divided by twice the length of the slide. The result is the surface tension of the liquid. The Wilhelmy method involves no rupture of the surface, and the surface is disturbed to a very small extent. This method is highly recommended. The author

<sup>9</sup> W. D. Harkins and H. F. Jordan, *J. Am. Chem. Soc.* 52, 1751 (1930).

<sup>10</sup> B. B. Freud and H. Z. Freud, *J. Am. Chem. Soc.* 52, 1772 (1930).

<sup>11</sup> A. E. Hauser, H. E. Edgerton, B. M. Holt, and J. I. Cox, Jr., *J. Phys. Chem.* 40, 973 (1936).

<sup>12</sup> M. Dole and J. A. Swartout, *J. Am. Chem. Soc.* 62, 3039 (1940).

<sup>13</sup> L. Wilhelmy, *Ann. Physik* 119, 177 (1863).

<sup>14</sup> D. G. Dervichian, *J. phys. radium* 6, 221, 429 (1935). See also W. D. Harkins and T. F. Anderson, *J. Am. Chem. Soc.* 59, 2189 (1937).



has found it to be of special value in the measurement of the surface tension of protein solutions and of other surface-active colloidal materials. A requirement of this method is that the slide must be completely wet by the liquid (zero contact angle).

*The drop-weight method* depends on the principle that the surface tension along with the density of a liquid determines the size of a drop forming at the end of a capillary. The larger the surface tension, the bigger the drop is before it breaks from the tip of the capillary and falls. These simple considerations yield the following equation:

$$\sigma = \frac{wg}{2\pi r} \quad (2)$$

where  $w$  is the weight of the drop in grams,  $g$  is the acceleration of gravity, and  $r$  is the radius of the capillary tip. The trouble is that not all the drop on the tip of the capillary falls. In addition to this complication Hauser<sup>15</sup> has found by means of ultra fast motion pictures that, when such a drop falls, it is accompanied by a number of smaller drops. These difficulties lead to serious errors if equation 2 is used to calculate the surface tension. Harkins and Brown<sup>16</sup> found empirically that a more accurate expression for surface tension as determined by the drop-weight method is

$$\sigma = \frac{wg}{2\pi f(r, V^{1/3})} \quad (3)$$

where  $V$  is the volume of the drop and  $f$  indicates that the surface tension is a function of  $r/V^{1/3}$ . Harkins and Brown studied the corrections to be applied, and their paper should be consulted. The drop-weight method is probably the least satisfactory of the common methods for determining surface tension.

Other satisfactory methods for the determination of surface tension are the pendent-drop method,<sup>17</sup> the sessile-drop method,<sup>18,19,20</sup> and Sugden's maximum-bubble-pressure method.<sup>21,22</sup>

<sup>15</sup> H. E. Edgerton, E. A. Hauser, and W. B. Tucker, *J. Phys. Chem.* 41, 1017 (1937).

<sup>16</sup> W. D. Harkins and F. E. Brown, *J. Am. Chem. Soc.* 41, 499 (1919).

W. D. Harkins, *Nature* 117, 690 (1926).

<sup>17</sup> J. M. Andreas, E. A. Hauser, and W. B. Tucker, *J. Phys. Chem.* 42, 1001 (1938).

<sup>18</sup> A. W. Porter, *Phil. Mag.* 25, 752 (1938).

<sup>19</sup> H. V. Tartar, V. Sivertz, and R. E. Reitmeier, *J. Am. Chem. Soc.* 62, 2375 (1940).

<sup>20</sup> O. L. Wheeler, H. V. Tartar, and E. C. Lingafelter, *J. Am. Chem. Soc.* 67, 2115 (1945).

<sup>21</sup> S. Sugden, *J. Chem. Soc.* 121, 858 (1922).

<sup>22</sup> T. H. Hazlehurst, *J. Chem. Education* 19, 61 (1942).



SURFACE ACTIVITY

Table 1 shows the values of the surface tension of some common liquids.

TABLE 1

SURFACE TENSION OF SOME COMMON LIQUIDS IN DYNES PER CENTIMETER AGAINST AIR

Substance	Temperature, °C.	Surface Tension
Acetic acid	20	27.6
	50	24.7
Acetone	20	23.7
	60	18.6
Benzene	20	28.88
	50	25.0
<i>n</i> -Butyric acid	20	26.8
	50	24.0
Ethanol	20	22.3
	50	19.8
Methanol	20	22.6
	50	20.1

Table 2 gives the surface tension of water in dynes per centimeter against air at various temperatures.

TABLE 2

SURFACE TENSION OF WATER IN DYNES PER CENTIMETER AGAINST AIR

Temperature, °C.	Surface Tension
0	75.6
10	74.22
15	73.49
18	73.05
20	72.75
25	71.97
30	71.18
40	69.56
50	67.91
60	66.18

SURFACE TENSION AND VAPOR PRESSURE

The surface of a liquid may be convex (drops of liquid), concave (liquid inside a capillary tube), or plane. These situations are diagrammed in Fig. 2.

In *A* the molecules of the surface are attracted by other liquid molecules to a less extent than they are in a plane surface; in *C* the surface molecules are attracted to a greater extent by other liquid molecules than they are in a plane surface. We would expect, therefore, that the vapor pressure of the liquid in a convex surface would be greater than the normal vapor pressure of the liquid (plane surface). On the other hand, the vapor pressure of a liquid with a concave surface should be less than the normal vapor pressure.

These expectations are fulfilled, and the relation is quantitatively expressed by Thomson's <sup>23</sup> equation

$$P = P_0 + \frac{2\sigma P_0 M}{RT\rho r} \quad (4)$$

where  $P_0$  is the normal vapor pressure of the liquid,  $\sigma$  is the surface tension,  $M$  is the molecular weight of the vapor,  $R$  is the gas constant which must be expressed in ergs per degree per mole ( $8.31 \times 10^7$ ),  $T$  is the absolute temperature,  $\rho$  is the density of the liquid, and  $r$  is the radius of the drop of

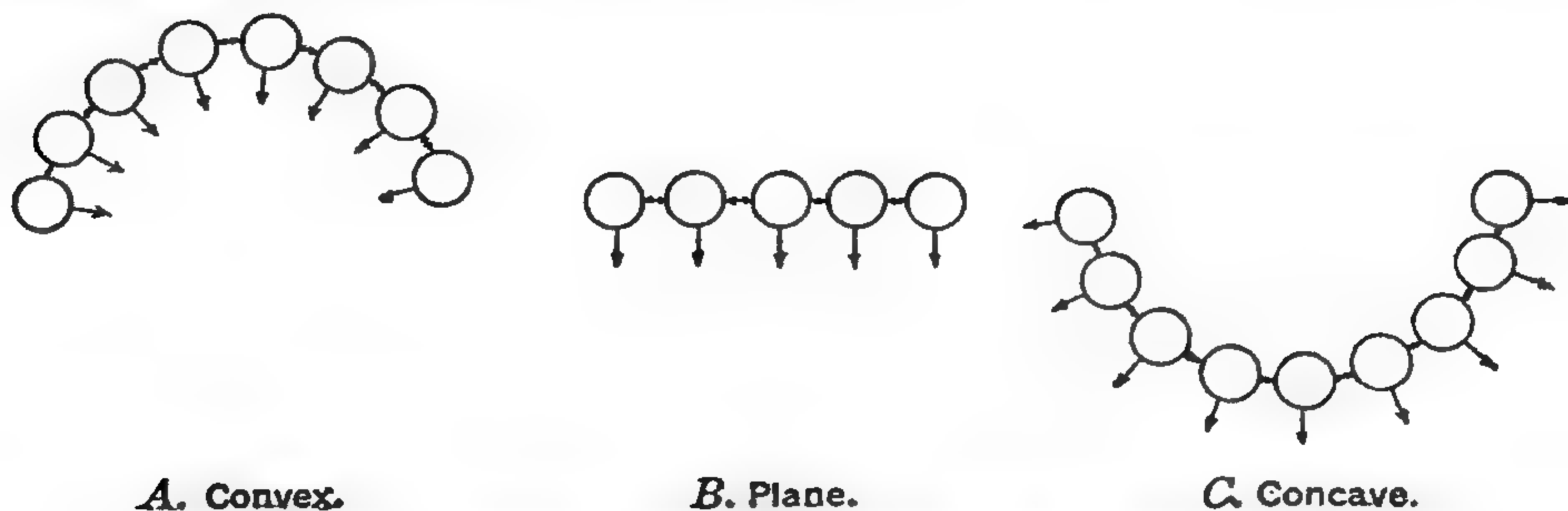


FIG. 2. Convex, plane, and concave liquid surfaces.

liquid or the radius of the capillary. For a convex surface,  $r$  is positive; for a concave surface, it is negative. With water at  $25^\circ$  C. equation 4 reduces to

$$P = 23.756 + \frac{25 \times 10^{-7}}{r} \quad (5)$$

For a capillary with a radius of  $10^{-6}$  cm. the vapor pressure of water would be reduced about 10 per cent.

## ENERGY OF A SURFACE

The total energy of a surface is made up of two terms, the free surface energy and the heat energy. The free surface energy is evidently equal to the surface tension in ergs per square centimeter multiplied by the total area of the surface. The heat term is defined as the heat that must be supplied to an expanding surface to maintain a constant temperature in the surface. The entire surface energy per unit area is the sum of these terms, or

$$u = \sigma - T \frac{d\sigma}{dT} \quad (6)$$

where  $d\sigma/dT$  is the rate of change of the surface tension with a change of temperature. This differential ( $d\sigma/dT$ ) is always negative; that is to say,

<sup>23</sup> W. Thomson, *Phil. Mag.* 42, 141, 448 (1871).

surface tension always decreases with increasing temperature. This negative sign cancels the negative sign in equation 6 to give a sum.

The heat energy required to expand the surface of water by one square centimeter at 20° C. is 45.7 ergs. The total energy required, therefore, is 72.75 plus 45.7 or is 118.5 ergs per square centimeter.

According to Eötvös, and to Ramsay and Shields, the surface tension is the following function of temperature

$$\sigma(MV)^{2/3} = K(T_c - T - 6) \quad (7)$$

where  $(MV)^{2/3}$  is proportional to the molecular surface,  $M$  is the molecular weight, and  $V$  is the specific volume of the liquid.  $T_c$  is the critical temperature of the liquid, and  $T$  is the temperature at which the surface tension is measured. These temperatures are expressed in degrees centigrade.  $K$  is a constant having the value 2.12 for normal unassociated liquids. If the liquid is associated, the constant is less than 2.12; i.e., the molecular weight is larger than that used in the calculation based on the assumption of no association.

#### ADDITION OF A SOLUTE

A substance dissolved in a liquid will do one of two things to the surface tension of that liquid: it will either raise or lower it. If the addition of the solute raises the surface tension, the solute is said to be capillary inactive; if it lowers the surface tension, the solute is capillary active. Most inorganic electrolytes in appreciable concentrations are capillary inactive. Soaps, proteins, and a great many other organic compounds are capillary active.

A little reflection will reveal that there must be a relation between the surface-tension raising or lowering and the amount of solute adsorbed. We have seen that the surface tension is a measure of the work required to bring molecules from the interior of the liquid to the surface. If positive adsorption has taken place, it means that less work is required to take a solute molecule to the surface than a solvent molecule and, accordingly, the surface tension of the solution must be less than that of the pure solvent. This reasoning is exactly expressed by Gibbs' adsorption equation, which may be derived as follows:

We have seen in Chapter 2, equation 37, that for a solution in equilibrium and at constant temperature and pressure

$$n_1 d\mu_1 + n_2 d\mu_2 = 0 \quad (8)$$

where  $n_1$  is the number of moles of solvent ( $H_2O$ ) and  $n_2$  is the number of moles of solute.  $\mu_1$  and  $\mu_2$  are the corresponding chemical potentials of the constituents. If we consider the surface of the solution as a separate phase,



equation 8 takes the form

$$S d\sigma + n_1' d\mu_1' + n_2' d\mu_2' = 0 \quad (9)$$

where  $S$  is the extent of the surface of the solution and the primes indicate that the functions apply to the surface. Both equation 8 and equation 9 state that for a system in equilibrium the net change of free energy is zero. At equilibrium the chemical potential of the water in bulk must equal that in the surface and correspondingly the chemical potential of the solute in the two phases are equal. If the chemical potentials are equal, then their differentials must be equal and we can identify  $d\mu_1$  with  $d\mu_1'$  and  $d\mu_2'$  with  $d\mu_2$ . Eliminating  $d\mu_1$  and  $d\mu_1'$  between equations 8 and 9 and rearranging

$$-\frac{S d\sigma}{d\mu_2} = n_2' - \frac{n_1' n_2}{n_1} \quad (10)$$

Now  $n_2'$  is the number of moles of the solute associated with  $n_1'$  moles of water in the surface phase.  $n_1'/n_1$  is the ratio of the number of moles of water in the surface to that in the bulk, and, if we multiply this ratio by the number of moles of solute,  $n_2$ , in the bulk phase, it will give the number of moles of solute in the surface phase, provided that the composition of the surface phase and bulk phases are identical. The terms on the right side of equation 10, therefore, represent the excess number of moles of solute in the surface phase of area  $S$ . The surface excess is the amount of solute adsorbed and

$$a = -\frac{S d\sigma}{d\mu_2} \quad (11)$$

where  $a$  is the number of moles of solute adsorbed. Since the chemical potential is related to the activity by

$$\mu = \mu_0 + RT \ln A \quad (12)$$

where  $A$  is the activity of the solute, then

$$d\mu = RT d \ln A \quad (13)$$

and, substituting equation 13 in equation 11, we have the Gibbs adsorption equation for one square centimeter of surface

$$a = -\frac{d\sigma}{RT d \ln A} = -\frac{A}{RT} \frac{d\sigma}{dA} \quad (14)$$

and for dilute solutions where the activity is very nearly equal to the concentration equation 14 can be written

$$a = -\frac{c}{RT} \frac{d\sigma}{dc} \quad (15)$$

where  $c$  is the concentration expressed in moles per liter.

We can see from equation 15 that, if the surface tension decreases with increasing concentration, the differential  $d\sigma/dc$  is negative and, accordingly,  $\alpha$  is positive and the concentration of the solute in the surface is greater than in the bulk phase. On the other hand, if  $\sigma$  increases with increasing concentration of solute in solution,  $\alpha$  is negative and the concentration of the solute in the surface phase is less than it is in the bulk phase. As has been noted, inorganic electrolytes, in general, increase the surface tension of water and are, accordingly, negatively adsorbed at a water surface.

Gibbs' adsorption equation has been tested in an ingenious manner by McBain and Humphrey.<sup>24</sup> The apparatus employed consisted of a microtome blade attached to the front of a small carriage. The carriage was provided with wheels and traveled at 35 feet per second over the surface of the solution, which was contained in a long trough. The microtome blade sliced off the top layer of the surface to a depth of 0.05 to 0.1 mm. The top layer of the solution was thus collected and analyzed, and its concentration was compared with that in the bulk of the solution. Their results showed satisfactory agreement with Gibbs' adsorption equation.

Aniansson and Lamm<sup>25</sup> have studied the adsorption of a sulfated detergent labeled with radioactive sulfur. They were able to measure the extent of adsorption by determining the amount of radioactivity on the surface. Their experiments serve to confirm the Gibbs adsorption equation.

Szyszkowski<sup>26</sup> proposed the following empirical equation to describe the surface-tension lowering produced by the addition of capillary-active materials

$$\frac{\sigma_0 - \sigma}{\sigma_0} = B \log \left( \frac{C}{A} + 1 \right) \quad (16)$$

where  $\sigma_0$  is the surface tension of the pure liquid and  $\sigma$  is the surface tension of the solution,  $C$  is the concentration of the solute in moles per liter of solution, and  $A$  and  $B$  are constants. If we differentiate the Szyszkowski equation and convert to natural logarithms, we have

$$\frac{d\sigma}{dC} = - \frac{0.434B\sigma_0}{C + A} \quad (17)$$

and for very dilute solutions equation 17 reduces to

$$\frac{d\sigma}{dC} = - \frac{0.434B\sigma_0}{A} \quad (18)$$

<sup>24</sup> J. W. McBain and C. W. Humphrey, *J. Phys. Chem.* **36**, 301 (1932).

<sup>25</sup> G. Aniansson and O. Lamm, *Nature* **165**, 357 (1950).

<sup>26</sup> B. von Szyszkowski, *Z. physik. Chem.* **64**, 385 (1908).

The initial slopes obtained from a plot of  $\sigma$  against  $C$  should yield a measure of the capillary activity of a solute. Shown in Table 3 are the initial slopes of a series of fatty acids.<sup>27</sup>

TABLE 3

INITIAL SURFACE TENSION-CONCENTRATION SLOPES FOR A SERIES OF FATTY ACIDS AT 20° C.

Fatty Acid	Number Carbon Atoms	Molecular Volume in (Å) <sup>3</sup>	Initial Slope in dynes/cm./moles/cm. <sup>3</sup>
Butyric	4	142	$-0.162 \times 10^6$
Valeric	5	169	$-0.600 \times 10^6$
Caproic	6	193	$-1.62 \times 10^6$
Heptoic	7	219	$-6.5 \times 10^6$
Caprylic	8	246	$-30 \times 10^6$
Pelargonic	9	268	$-101 \times 10^6$
Capric	10	297	$-530 \times 10^6$

It can be seen from Table 3 that the ratio of the initial surface tension-concentration slope of a fatty acid to the next lower homologue is very nearly constant throughout the series and is equal to 3.72. This regularity was first noted by Traube<sup>28</sup> and is known as Traube's rule.

The theoretical significance of Traube's rule was first considered by Langmuir.<sup>29</sup> The initial slope of the surface tension-concentration curve is evidently proportional to the distribution of the solute in the surface relative to that in the bulk phase. From the Boltzmann distribution equation, the relation between the initial slope for a fatty acid and the next-higher homologue should be

$$\frac{\text{Slope}_n}{\text{Slope}_{n+1}} = e^{(\lambda_1 - \lambda_2)/kT} \quad (19)$$

where  $\lambda_1$  and  $\lambda_2$  are the energies in ergs required to remove the fatty acids from solution and place them on the surface.  $k$  is Boltzmann's constant, and  $T$  is the absolute temperature. We have noted that the ratio of initial slopes from Table 3 is 3.72. Substituting this value in equation 19, we find at 20° C. that  $5.28 \times 10^{-14}$  erg per molecule or 760 calories per mole represents the free energy decrease per CH<sub>2</sub>-group in transferring a fatty acid from solution to the surface.

Ward<sup>27</sup> has further considered the theoretical implications of Traube's rule and points out that the rule is consistent with the idea that the fatty acids

<sup>27</sup> A. F. H. Ward, *Trans. Faraday Soc.* 42, 399 (1946).

<sup>28</sup> Traube, *Liebigs Ann. Chem.* 265, 27 (1891).

<sup>29</sup> I. Langmuir, *J. Am. Chem. Soc.* 39, 1848 (1917).



exist both in solution and in the surface at low concentrations as random coils which approach the form of spheres.

#### SURFACE TENSION OF HIGH-MOLECULAR-WEIGHT COMPOUNDS

The surface tension of solutions of colloidal surface-active substances present several complicated features. For one thing, the time required for attainment of equilibrium in many substances is very long, and it is really doubtful that in some a true equilibrium tension is ever observed. Hauser and Swearingen<sup>11</sup> investigated the aging of the surfaces of egg albumin; their results show that the final surface tension values are approached very slowly.

The slow attainment of the final value for the surface tension of such solutions is definitely not due to the slow rate of diffusion of the protein molecules to the surface. It has been calculated<sup>12</sup> that, if every egg albumin molecule that diffused to the surface remained in the surface, the surface of a 0.05 per cent egg albumin solution would be completely saturated in 0.58 second. There are several possible reasons for the slow approach to equilibrium. Possibly one of the most important is that the native egg albumin is really capillary inactive, and it is only after the native molecule has been surface-denatured that it becomes capillary active. The transfer of the native molecule into the surface where it can surface-denature encounters an energy barrier which must be overcome before the denatured, capillary-active egg albumin can be formed. This requires time.

With proteins like egg albumin, it is doubtful that an equilibrium exists between the denatured molecules in the film and the protein solution; the denatured molecules are so insoluble that none of them can return to the liquid. Therefore it is meaningless to attempt to apply Gibbs' adsorption equation to such a system.

Hydrogen ions, in general, have a pronounced effect upon the surface tension of proteins, a minimum in the surface tension being observed in the isoelectric zone.

Ross<sup>13</sup> has given a general summary of the influence of the rate of diffusion of the solute molecules to the surface and the relation of this rate to the surface tension.

Some substances such as soaps and long-chain sulfated alcohols exhibit a distinct minimum in the surface tension-concentration curve. However, Miles and Shedlowsky<sup>14</sup> attribute such minima to impurities and find that pure surface-active solutes do not show these minima.

<sup>11</sup> E. A. Hauser and L. E. Swearingen, *J. Phys. Chem.* **45**, 644 (1941).

<sup>12</sup> H. Neurath and H. B. Bull, *Chem. Revs.* **23**, 391 (1938).

<sup>13</sup> S. Ross, *J. Am. Chem. Soc.* **67**, 990 (1945).

<sup>14</sup> G. D. Miles and L. Shedlowsky, *J. Phys. Chem.* **48**, 57 (1944).

G. D. Miles, *J. Phys. Chem.* **49**, 71 (1945).

Du Noüy<sup>34</sup> and other workers have reported rather sharp maxima and minima of the surface tension of protein solutions as the protein concentration is increased. They have attempted to calculate molecular dimensions from these maxima and minima on the assumption that the irregularities in the surface tension are due to different molecular orientations. The author is of the opinion that these maxima and minima are artifacts and that this attempt to evaluate molecular dimensions is completely in error.

### INTERFACIAL TENSION

Up to this point we have confined ourselves to consideration of the solution-air surface. In general, the same treatment applies to a liquid-liquid interface. The interfacial tension is a measure of the energy required to bring molecules from within one liquid to the interface plus the energy needed to take molecules from the other liquid and put them in the interface. The interfacial tension is, in general, much lower than the sum of the surface tensions of the two immiscible liquids. The reason is that the potential energy of a molecule at the interface is lower than at the air-liquid surface. This, in turn, is due to the fact that more of the attractive forces of the molecules have been satisfied at an interface than at a liquid-air surface. The relation between the interfacial tension and the surface tensions of the two liquids is given by the approximate Antonoff's rule,

$$\sigma_{AB} = \sigma_A' - \sigma_B' \quad (20)$$

where  $\sigma_A'$  is the surface tension of liquid *A* saturated with liquid *B*, and  $\sigma_B'$  is the surface tension of liquid *B* saturated with *A*. For a critical discussion of Antonoff's rule, see Harkins.<sup>35</sup>

Harkins and Zollman,<sup>36</sup> as well as Ward and Tordai,<sup>37</sup> have described methods for the determination of interfacial tensions. These techniques are, in principle, the same as those used for the measurement of surface tension, but the determinations are usually somewhat more troublesome.

### SURFACES OF LIVING CELLS

The interfacial tension of some living cells has been determined, and Harvey<sup>38</sup> states two general conclusions that can be drawn from such measurements as have been made: (1) the tension is very low, less than 1 dyne per centimeter; and (2) the cell surface has elastic properties. The

<sup>34</sup> P. L. du Noüy, *Surface Equilibria of Biological and Organic Colloids*, The Chemical Catalog Co., Inc., New York, 1926.

<sup>35</sup> W. D. Harkins, *Proc. Natl. Acad. Sci. U. S. A.* 5, 569 (1919).

<sup>36</sup> W. D. Harkins and H. Zollman, *J. Am. Chem. Soc.* 48, 69 (1926).

<sup>37</sup> A. F. H. Ward and L. Tordai, *J. Sci. Instruments* 21, 143 (1944).

<sup>38</sup> E. N. Harvey, *Trans. Faraday Soc.* 33, 943 (1937).



centrifuge method can be used for egg cells which contain oil globules (oil globules are less dense than the cell fluid) and yolk granules (yolk granules are more dense than the cell fluid). In this method the cells are placed in a centrifuge microscope, and the centrifugal force just sufficient to separate the cell into two halves is determined. This force is equated to the interfacial tension forces around the circumference of the cell, and the interfacial tension is calculated.

Another method used by Cole<sup>39</sup> is to measure the flattening of the cell produced by a microbeam of gold  $6\ \mu$  thick and  $180\ \mu$  wide. The cell was photographed with and without the microbeam, and the corresponding cell radii were measured. From the flattening of the cell by the microbeam, the interfacial tension of the cell was calculated. It was found that the cell surface of the unfertilized *Arbacia* egg was elastic with a very low tension. When the tensions were extrapolated to the uncompressed state, a value of 0.08 dyne per centimeter was found. The fertilized *Arbacia* egg with its fertilization membrane was, however, quite rigid, and no measurement of the interfacial tension was possible.

Ascherson,<sup>40</sup> as long ago as 1840, suggested that cell membranes were analogous to the "skin" which forms around oil droplets suspended in a solution of "albumin" (protein). It is entirely possible that the structural element of animal cell membranes is indeed fiber protein which gives the cell surface its elastic properties. Parpart and Dziemian,<sup>41</sup> for example, have analytical evidence that the mammalian red blood cell membrane contains a collagen-like protein.

### SOLID-LIQUID INTERFACE

If a drop of liquid is placed on a solid surface, the liquid may spread over the solid and completely wet it, or it may remain as a drop. The situation is diagrammed in Fig. 3.

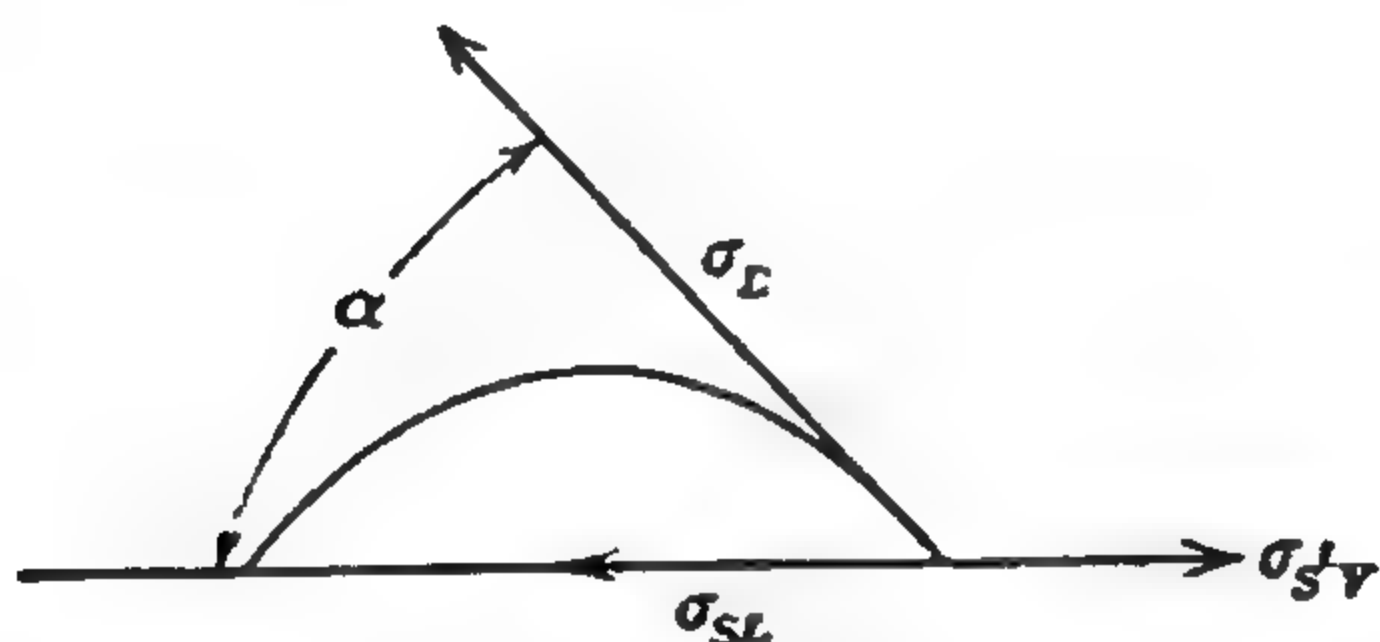


FIG. 3. The resolution of forces acting on a drop of liquid at a solid surface.

The angle  $\alpha$  is called the contact angle. The magnitude of this angle depends on the resultant of the forces acting along the surface of the solid. That is,

$$\sigma_{S'V} = \sigma_{SL} + \sigma_L \cos \alpha \quad (21)$$

where  $\sigma_{S'V}$  is the surface tension of the solid saturated with the vapor of

<sup>39</sup> K. S. Cole, *J. Cellular Comp. Physiol.* 1, 1 (1932).

<sup>40</sup> F. M. Ascherson, *Arch. Anat. u. Physiol.* 1840, 44.

<sup>41</sup> A. K. Parpart and A. J. Dziemian, *Cold Spring Harbor Symposium Quant. Biol.* 8, 17 (1940).



the liquid,  $\sigma_L$  is the surface tension of the liquid, and  $\sigma_{SL}$  is the interfacial tension of the solid-liquid interface.

The determination of the contact angle is evidently of great importance in the evaluation of the wetting quality of a surface. A large number of techniques have been devised for the measurement of contact angles. The fact that so many techniques have been employed indicates the difficulty of such measurements. For one thing, the receding and advancing angles are usually not equal to each other. Surface contaminations influence the magnitude very profoundly. The solid may also undergo slow changes due to the presence of the liquid. For example, a fresh surface of solid paraffin wax is quite hydrophobic with a large contact angle against water (about  $105^\circ$ ). If, however, the paraffin wax is immersed in water for several days, the surface becomes hydrophilic and gives a contact angle of zero or almost zero. Harkins<sup>42</sup> recommends the use of a tilting plate. Bartell and Bristol<sup>43</sup> have devised a suitable method.

There is considerable confusion in the literature in regard to the term adhesion tension. Harkins and Livingston<sup>44</sup> have considered the whole problem of wetting of solids by liquids and have pointed out in a precise manner the source of our confusion. We shall follow their treatment. The work required to separate a unit area of a liquid from a unit area of a solid is clearly

$$\text{Work of adhesion} = \sigma_{SA} + \sigma_L - \sigma_{SL} \quad (22)$$

where  $\sigma_{SA}$  is the surface tension of the solid in a vacuum. The source of confusion arose in attempting to identify  $\sigma_{SA}$  and  $\sigma_{S'V}$ . It is quite obvious that these two terms are very far from being identical. In general,  $\sigma_{S'V}$  is very much less than  $\sigma_{SA}$ . Equating these two terms, as was done in the literature, would allow us to substitute equation 21 into equation 22. We would then have per unit area

$$\text{Adhesion tension} = \sigma_L + \sigma_L \cos \alpha \quad (23)$$

This expression, however, is true only if we consider the solid surface saturated with vapor. The expression has been so misused that Harkins and Livingston propose that the term adhesion tension be abandoned.

They propose instead the term spreading coefficient ( $\pi$ ) which is defined as

$$\pi = \sigma_{S'V} - \sigma_{SL} = \sigma_L \cos \alpha \quad (24)$$

and represents the tendency for a liquid to spread over a solid saturated with the liquid vapor and to wet it.

<sup>42</sup> W. D. Harkins and F. M. Fowkes, *J. Am. Chem. Soc.* **60**, 1511 (1938); **62**, 3377 (1940).

<sup>43</sup> F. E. Bartell and K. E. Bristol, *J. Phys. Chem.* **44**, 86 (1940).

<sup>44</sup> W. D. Harkins and H. K. Livingston, *J. Chem. Phys.* **10**, 342 (1942).

## MUDD INTERFACIAL TECHNIQUE

Mudd and co-workers<sup>45</sup> have developed a technique for the study of the wetting characteristics of cell surfaces. The aqueous suspension of bacteria is placed on a microscope slide adjacent to a drop of oil. When a cover slip is lowered over this preparation, an oil-water boundary is formed which advances across the microscope field. When the boundary reaches a bacterium, one of three things may happen: (1) if the surface of the bacterium is hydrophilic, it remains in the water phase; (2) if the surface is hydrophobic (oilophilic), it passes easily into the oil phase; and (3) if it is neither pronouncedly hydrophilic or hydrophobic, it tends to remain at the oil-water interface.

Moyer<sup>46</sup> used this technique to determine the nature of the surface of latex particles from the various species of euphorbia and found that those particles with a low isoelectric point about pH 3 showed preferential wettability by oil, whereas those with higher isoelectric points about pH 5 had surfaces that were wetted by water. These findings were consistent with his electrophoretic studies in which he postulated that the particles with low isoelectric points had surfaces that were predominately sterol in character, whereas those with the higher isoelectric point had a covering of protein.

## MOLECULAR POLARITY

As a result of his studies on the surface tension lowering produced by straight-chain fatty acids and also from his experiments on spread monolayers, which we shall discuss presently, Langmuir was led to his picture of the orientation of polar molecules at surface. In Chapter 1, we treated polar molecules from the standpoint of their electrical asymmetry, the dipole moment being a measure of this asymmetry. The concept of polarity suggested here is essentially one of solubility. Hydrocarbons are characteristically insoluble in water. If groups such as  $-\text{COOH}$ ,  $-\text{OH}$ ,  $-\text{NH}_2$ , and  $-\text{SO}_3\text{H}$  are substituted into a hydrocarbon, they tend to make the hydrocarbon water soluble. Such groups are called hydrophilic groups (water-loving). It will be recognized that the above polar groups are capable of binding water through hydrogen bonds. The hydrocarbon portion is said to be hydrophobic (water-hating). If the hydrocarbon chain is small and the attraction of the hydrophilic group of water strong enough, the resulting molecule will be completely water soluble as, for example, acetic and propionic acids. At a water-air surface such polar molecules are situated with the polar group (hydrophilic group) sticking into the water and with the hydrocarbon chain directed towards the air. At higher con-

<sup>45</sup> S. Mudd and E. B. H. Mudd, *J. Exptl. Med.* 40, 633 (1924).

<sup>46</sup> L. S. Moyer, *Am. J. Botany* 22, 609 (1935).



centration of adsorbed fatty acids the hydrocarbon chain is oriented vertically to the surface.

We have used the word hydrophobic in describing the hydrocarbon part of a molecule. It must not be imagined that there is an actual repulsion between water and a hydrocarbon chain; there indeed exist fairly large attractive forces between water and the hydrocarbon, but it so happens that these forces are smaller than those existing between the water molecules, and the paraffin molecules are unable to break the water-water bond, and, accordingly, hydrocarbons are insoluble in water.

If a drop of water be placed on a clean paraffin surface, it will be found that the contact angle is about 105°. If there were no attraction between the water and paraffin, no wetting would occur and the contact angle would be 180°. It is possible to calculate the total energy in ergs required to separate an interface 1 sq. cm. in area at 20° for the combinations of paraffin and paraffin, paraffin and water, and water and water. The results of these calculations are shown below.

<i>n</i> -butane	<i>n</i> -octane	<i>n</i> -octane	water
92	100	107	233
<i>n</i> -butane	<i>n</i> -octane	water	water

As shown above, the attraction of water for *n*-octane is greater than octane for octane, and, accordingly, the term “hydrophobic” when used in this connection is definitely a misnomer. The energy of the “bonds” between *n*-octane molecules can be approximately calculated from the results given above and turns out to be about 6 kilocalories per mole; a not inconsiderable magnitude. The attraction between paraffin chains involves the so-called van der Waals or London forces, which were discussed to some extent in Chapter 1. These forces arise from induced dipoles. Aliphatic hydrocarbons have no permanent dipole moment; nevertheless, on a time average the electrons in atoms will suffer a temporary displacement, giving rise to a momentary dipole. Such a transitory dipole is capable of inducing a dipole in a neighboring atom and thus giving rise to an attractive force. The tendency of an atom to form temporary dipoles is related to the polarizability of the atom which in turn is related to the index of refraction to light. The theoretical treatment of the van der Waals forces or, as they are sometimes called, secondary valence bonds, is fairly complex, and their magnitudes cannot, in general, be calculated exactly. The importance of such forces for biological structures appears to be very great and are easily as significant as hydrogen bonds.

ENERGY OF ADSORPTION

The free energy required to transfer molecules from the vapor state to the solid surface is clearly a quantitative measure of the affinity of the solid



for the vapor. As pointed out by Dole and McLaren,<sup>47</sup> this transfer can be conducted in either one of two ways. For example, we can transfer water molecules in the vapor state at pressure  $P$  to the solid or we can transfer water molecules from pure water to the vapor at  $P_0$  and then to the solid surface. The free energy for the first process is

$$\Delta F_1 = -RT \int_0^1 n d \ln \frac{P}{P_0} \quad (25)$$

where  $n$  is the number of moles of water adsorbed by the solid. The free energy associated with the second process is

$$\Delta F_2 = -RT \int_0^n \ln \frac{P}{P_0} dn \quad (26)$$

In the first process  $P/P_0$  is considered the independent variable, whereas in the second process  $n$  is the independent variable. The second process involves the free energy of expanding the water vapor from pressure  $P_0$  to pressure  $P$ . It can be shown that the free energy change of these two processes is related by

$$\Delta F_2 - \Delta F_1 = nRT \ln \frac{P}{P_0} \quad (27)$$

Evidently, for complete saturation of the surface the total free energy change for the two processes is identical since at saturation  $P$  is equal to  $P_0$ .

In order to integrate equation 25,  $nP_0/P$  is plotted against  $P/P_0$  and the area under the curve determined. Unfortunately, it is difficult to measure the area under the curve because for small values of  $P/P_0$  the curve swings upward too steeply and appears to approach infinity when  $P/P_0$  is equal to zero. To circumvent this difficulty a modification of the method used by Boyd and Livingston<sup>48</sup> is suggested.

The region under the curve which results from a plot of  $nP_0/P$  against  $P/P_0$  is divided into segments by drawing vertical lines at regular and convenient intervals to the  $P/P_0$ -axis. Successive areas under the curve to right and to left of  $P/P_0 = 0.5$  are measured with a planimeter. The area under the curve from  $P/P_0 = 0$  to  $P/P_0 = 0.5$  cannot, of course, be measured with a planimeter, and indeed it is this area which we are seeking and have resorted to this complicated procedure to obtain. The areas to the left of the mid-vertical line are given negative signs and those to the right are assigned positive signs. These areas are then plotted against  $P/P_0$ , and the resulting curve is extrapolated to  $P/P_0 = 0$  and  $P/P_0 = 1$ . The

<sup>47</sup> M. Dole and A. D. McLaren, *J. Am. Chem. Soc.* **69**, 651 (1947).

<sup>48</sup> G. E. Boyd and H. K. Livingston, *J. Am. Chem. Soc.*, **64**, 2383 (1942); see especially H. K. Livingston, Ph. D. thesis, University of Chicago, 1941.

free energy of adsorption to any given relative vapor pressure can be found by adding the ordinate corresponding to  $P/P_0$  to the ordinate found by extrapolation to  $P/P_0 = 0$  and multiplying this sum by  $RT \cdot M$ . The values of  $n$ , the amount of vapor adsorbed, corresponding to a series of  $P/P_0$  values are then obtained from the adsorption isotherm where the amount adsorbed is plotted against the relative vapor pressure. This allows us to plot  $\Delta F$  against amount of vapor adsorbed. If  $\Delta F$  is calculated for the solid in the presence of its saturated vapor, we have a direct measure of the difference  $(\sigma_{SA} - \sigma_{S'V})$ , and, provided that we know the surface area of the solid, we can express  $\Delta F$  in terms of this surface-tension difference. Practically, in order to study the amount of vapor adsorbed by a solid, a large surface is necessary; otherwise the weight of the vapor adsorbed will be so small that accuracy will be impossible. Finely powdered or porous solids are employed.

Table 4 shows the integral free energies in calories for the adsorption of water vapor on 100 grams of dry protein, along with other information that we shall discuss presently.<sup>49</sup>

TABLE 4

CONSTANTS INVOLVED IN ADSORPTION OF WATER VAPOR BY 100 GRAMS OF DRY PROTEIN AT 25° C.

Protein	$\Delta F_{25}$	$C$	$a_1$	$a_8$
Silk	-656	12.78	4.07	33
Wool	-975	11.13	6.58	41
Collagen	-1620	17.80	9.52	62
Egg albumin, native	-956	11.60	6.15	51
heat-coagulated	-814	13.62	4.97	36
Serum albumin, horse	-1034	11.25	6.73	46
$\gamma$ -Globulin, horse	-1119	11.85	7.16	63

The heats of adsorption can be measured in a precision calorimeter as was done by Harkins and Jura<sup>50</sup> or, if the adsorption has been studied at two different temperatures as a function of vapor pressure, it can be calculated.<sup>51</sup> The isosteric heat of adsorption which is the partial molal heat of the adsorption can be calculated by the equation

$$-q = \frac{d \Delta H}{dn} = \frac{RT_1 T_2}{(T_2 - T_1)} \ln \frac{P}{P_0} \frac{P_0'}{P'} \quad (28)$$

$P_0'/P'$  is the reciprocal of the relative vapor pressure at the higher temperature  $T_2$  which produces the same moles of water adsorption as at the lower temperature  $T_1$ . The value of  $q$  may be obtained from equation 28

<sup>49</sup> H. B. Bull, *J. Am. Chem. Soc.* 66, 1499 (1944).

<sup>50</sup> W. D. Harkins and G. Jura, *J. Am. Chem. Soc.* 66, 919 (1944).

<sup>51</sup> S. Brunauer, *Adsorption of Gases and Vapors*, Princeton University Press, N. J., 1943.

by a graphical method. Adsorption isotherms at the two different temperatures are drawn, and the value of  $P'/P_0'$  at the upper temperature necessary to produce the same number of moles of adsorption as at the relative vapor pressure  $P/P_0$  of the lower temperature are determined. These values are then substituted in equation 28 to calculate  $q$ . The result is the differential heat of adsorption. If  $q$  be plotted against  $n$  and the area under the curve measured with a planimeter, the integral heat of adsorption is found. There is, however, likely to be an ambiguity associated with the portion of the curve at higher vapor pressures because the number of moles adsorbed at saturation are not, in general, equal at the two temperatures.

Davis and McLaren<sup>52</sup> have calculated the differential free energies, heats and entropies of adsorption of water by proteins as functions of the water adsorbed.

#### ADSORPTION FROM SOLUTION

A great deal of experimental and theoretical work has been done on adsorption both at solid-gas and at solid-liquid surfaces, and it is possible to consider only a small fraction of this published material.

Freundlich<sup>53</sup> proposed an empirical adsorption isotherm to describe the relation between the amount of solute adsorbed and its concentrations. His equation is

$$\frac{a}{m} = \alpha C^{1/n} \quad (29)$$

where  $a$  is the amount of solute adsorbed,  $m$  is the weight of the adsorbent in grams,  $C$  is the equilibrium concentration of the solute, and  $\alpha$  and  $1/n$  are constants. Equation 29 may be converted into logarithmic form

$$\log \frac{a}{m} = \log \alpha + \frac{1}{n} \log C \quad (30)$$

If Freundlich's equation is obeyed the plot of  $\log a/m$  against  $\log C$  should give a straight line. The slope of this line is equal to  $1/n$ , and the intercept on the  $\log a/m$  axis is equal to  $\log \alpha$ .

Langmuir<sup>54</sup> was able to derive an adsorption equation from theoretical consideration by equating the rate of evaporation of a gas from a solid surface to the rate of condensation.

The rate of condensation on a solid surface is proportional to  $\mu$ , the number of molecules striking each square centimeter of surface per second, and to  $(1 - \theta)$ , the fraction of the surface not covered by the adsorbed molecules, and to  $\phi$ , the fraction of the molecular collisions that are inelas-

<sup>52</sup> S. Davis and A. D. McLaren, *J. Polymer Sci.* 3, 16 (1948).

<sup>53</sup> H. Freundlich, *Kapillarchemi*, Akademische Verlagsgesellschaft, Leipzig, 1930.

<sup>54</sup> I. Langmuir, *J. Am. Chem. Soc.* 40, 1361 (1918).



tic. The rate of evaporation is equal to  $\nu\theta$ , where  $\nu$  is the rate of evaporation when all the surface is occupied and  $\theta$  is the fraction of the surface already occupied. When equilibrium is established the two rates are equal; that is

$$\mu\phi(1 - \theta) = \nu\theta \quad (31)$$

and

$$\theta = \frac{\phi\mu}{\nu + \phi\mu} \quad (32)$$

If  $X$  is the number of molecules per square centimeter at a given gas pressure and  $X_0$  is the number of molecules per square centimeter at saturation,

$$\theta = \frac{X}{X_0} \quad (33)$$

Substituting the value of  $\theta$  in equation 32 and rearranging, we have

$$X = \frac{X_0(\phi\mu/\nu)}{1 + (\phi\mu/\nu)} \quad (34)$$

Since  $\phi/\nu$  is constant and  $\mu$  is proportional to the gas pressure  $P$ , and  $X$  is proportional to the number of gram molecules adsorbed per gram of adsorbent, we have

$$a = \frac{\alpha_1\beta_1P}{1 + \alpha_1P} \quad (35)$$

where  $a$  is the amount of solute adsorbed and  $\alpha_1$  and  $\beta_1$  are the proportionality constants. We can also apply the Langmuir equation to the adsorption at a liquid-solid interface. In this case we replace pressure by concentration to obtain

$$a = \frac{\alpha\beta C}{1 + \alpha C} \quad (36)$$

Equation 36 can be rearranged to give

$$\frac{C}{a} = \frac{1}{\alpha\beta} + \frac{C}{\beta} \quad (37)$$

If  $C/a$  is plotted against  $C$ , a straight line should be obtained, the slope of which is  $1/\beta$  and the intercept is  $1/\alpha\beta$ . At concentrations sufficient to saturate the adsorbing surface, we know from equation 36 that

$$a = \frac{\alpha\beta C}{\alpha C} = \beta \quad (38)$$

from which we conclude that  $\beta$  has the same dimensions as  $a$ , which, as we realize, has the dimensions of concentration per unit area or  $L^{-2}$ . Sub-

stituting this dimension for  $\beta$  in equation 36, we find  $\alpha$  to be dimensionless;  $\alpha$  is related to the energy of adsorption.

The Langmuir adsorption equation has a very general form. Consider for example, a weak acid ionizing as



We have the expression for the ionization constant

$$K = \frac{\text{H}^+ \times \text{A}^-}{\text{HA}} \quad (39)$$

The concentration of the anion,  $\text{A}^-$ , is evidently equal to  $(\text{A}_0 - \text{HA})$  where  $\text{A}_0$  is the total amount of acid present in its unionized as well as ionized form. Substituting this value of  $\text{A}^-$  in equation 39 and rearranging we have

$$\text{HA} = \frac{\text{A}_0 \times \text{H}^+}{(K + \text{H}^+)} \quad (40)$$

which can still further be rearranged by multiplying the numerator and denominator by  $1/K$  to obtain

$$\text{HA} = \frac{1/K \times \text{A}_0 \times \text{H}^+}{(1 + 1/K \times \text{H}^+)} \quad (41)$$

Clearly, equation 41 is closely analogous to equation 36 where  $1/K$  is equivalent to  $\alpha$  and  $\text{A}_0$  is equivalent to  $\beta$ . Klotz and co-workers<sup>12</sup> have given a very elegant interpretation of binding of ions by proteins and of the relationship of the Langmuir adsorption equation to the mass-action law. They have also published a large number of papers dealing with the experimental determination of ion adsorption in a number of protein systems.

To illustrate their approach let us limit the discussion to a protein, each molecule of which is capable of binding two small ions or molecules, although it will be apparent that the same treatment could be extended to the binding of any number of ions, as indeed Klotz has done. We have then the two reactions



and



where  $P$  is the protein and  $M$  is the ion or small molecule being bound.

<sup>12</sup> I. M. Klotz, *Arch. Biochem.* 9, 109 (1946).

I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Am. Chem. Soc.* 68, 1486 (1946).

I. M. Klotz and H. G. Curme, *J. Am. Chem. Soc.* 70, 939 (1948).

I. M. Klotz, *Cold Spring Harbor Symposia Quant. Biol.* 14, 97 (1950).

The equilibrium constants for these reactions are

$$K_1 = \frac{PM}{P \times M} \quad (42)$$

and

$$K_2 = \frac{PM_2}{PM \times M} \quad (43)$$

and

$$K_1 K_2 = \frac{PM_2}{P \times M^2} \quad (44)$$

The ratio of moles of bound ion to total moles of protein is evidently

$$r = \frac{PM + 2PM_2}{P + PM + PM_2} \quad (45)$$

Substituting equations 42 and 43 into equation 45, eliminating  $P$ , and factoring  $M$  out of the numerator give

$$r = \frac{M(K_1 + 2K_1 K_2 M)}{1 + K_1 M + K_1 K_2 M^2} \quad (46)$$

$(K_1 + 2K_1 K_2 M)$  is evidently the derivative of  $(1 + K_1 M + K_1 K_2 M^2)$  with respect to  $M$ . Then

$$r = \frac{M d(1 + K_1 M + K_1 K_2 M^2)}{(1 + K_1 M + K_1 K_2 M^2)} \quad (47)$$

The Langmuir equation (equation 36) can be written as

$$r = \frac{MM_0}{K + M} \quad (48)$$

where  $M_0$  is the maximum number of moles of ions bound and  $K$  is an intrinsic dissociation constant (reciprocal of binding constant).

Equating expressions 47 and 48, we have

$$\frac{d(1 + K_1 M + K_1 K_2 M^2)}{1 + K_1 M + K_1 K_2 M^2} = \frac{M_0}{K + M} \quad (49)$$

The integration of equation 49 gives

$$1 + K_1 M + K_1 K_2 M^2 = \left(1 + \frac{M}{K}\right)^2 \quad (50)$$

Differentiating equation 50, there results

$$K_1 + 2K_1 K_2 M = \frac{2}{K} \left(1 + \frac{M}{K}\right) \quad (51)$$



Differentiation of equation 51 gives

$$2K_1K_2 = \frac{2}{K^2} \quad (52)$$

Solving for  $K_1$  and  $K_2$  in terms of  $K$ , we find

$$K_1 = \frac{2}{K} \quad (53)$$

and

$$K_2 = \frac{1}{2K} \quad (54)$$

and

$$\frac{K_1}{K_2} = 4 \quad (55)$$

Here we see that the ratio between the first and second association constant is four; a conclusion that was noted in Chapter 7 under the discussion of the ionization constants of dibasic acids and which can be arrived at in a simple manner. If the intrinsic affinities of two carboxyl groups in a dibasic organic acid for protons are equal to each other, the tendency to lose the first hydrogen would be twice as great as to lose the second. This is so because there are twice as many hydrogens bound when both carboxyls are unionized. On the other hand, the tendency to bind a hydrogen on one of two ionized carboxyls is twice that for binding of hydrogen after one of the carboxyl groups has taken up a hydrogen. Accordingly, the ratio of the two dissociation constants should be one to four, which is in accord with equation 55.

We can, as Klotz has done, generalize the expression for the relation between the individual association constants and the intrinsic dissociation constant to obtain

$$K_n = \frac{M_0 - (n - 1) \frac{1}{K}}{n} \quad (56)$$

where  $n$  is the intermediate number of ions bound on the protein molecule and allows us to evaluate any one of the binding constants from the intrinsic constant.

The formulation given above is valid only if the adsorption of the first ion does not influence the adsorption of a second ion except to the extent that there is one less site to which the second ion can attach itself. If equation 48 be reduced to the form

$$\frac{1}{r} = \frac{K}{M_0} \frac{1}{M} + \frac{1}{M_0} \quad (57)$$

and  $1/r$  be plotted against  $1/M$  and a straight line obtained, then Langmuir's equation applies, and, further, there is no interaction between the ions as they are adsorbed.

Ions that are successively bound do frequently interact. For example, Table 3, Chapter 7, shows that the ratio between the first and second ionization constants of the dicarboxylic acids is larger than 4. This means that electrostatic interaction is considerable and can greatly outweigh the statistical effect. There can likewise be van der Waals interaction between organic ions; this, no doubt, occurs in the adsorption of sulfated detergent by protein.

The plot of equation 57, as indicated, is a rather insensitive way of handling the experimental data and tends to obscure the experimental variations.

Scatchard<sup>56</sup> has suggested that a more appropriate plot is to rearrange equation 48 and solve for  $M$  to yield

$$M = \frac{rK}{M_0 - r} \quad (58)$$

Multiplying this equation by  $\frac{M_0 - r}{M}$  gives

$$(M_0 - r) = \frac{rK}{M} \quad (59)$$

Plotting  $r/M$  against  $r$  gives a straight line if  $K$  is a constant. The intercept on  $r/M$ -axis is  $M_0/K$  and the intercept on the  $r$ -axis is  $M_0$ . Figure 4 illustrates the two types of plots to determine the maximum number of calcium ions bound by casein.

Often  $M_0$  does not approach a limiting value with increasing ion concentration, and it seems probable that at least one reason for this ambiguity arises from the fact that ions of opposite sign are being adsorbed, the total number of ions adsorbed thus tending to be increased.

Karush and Sonenberg<sup>57</sup> and Karush<sup>58</sup> have preferred to interpret the binding of anions by bovine serum albumin in terms of two groups of binding sites, each group characterized by a particular value of the intrinsic association constant. The adsorption equation in this event becomes

$$\frac{r}{M} = \frac{M_0'K_1}{1 + K_1M} + \frac{M_0''K_2}{1 + K_2M} \quad (60)$$

<sup>56</sup> G. Scatchard, *Ann. N. Y. Acad. Sci.* 51, 660 (1949).

<sup>57</sup> F. Karush and M. Sonenberg, *J. Am. Chem. Soc.* 71, 1369 (1949).

<sup>58</sup> F. Karush, *J. Am. Chem. Soc.* 72, 2705 (1950).

They calculate at 25° C. in 0.05 *M* phosphate buffer at pH 7.0 that  $M_0'$  is 4.66 and  $M_0''$  is 17.34 while  $K_1$  is  $6.16 \times 10^4$  and  $K_2$  is  $0.19 \times 10^4$  for the anionic dye, *p*-(2-hydroxy-5-methylphenylazo)-benzoic acid.

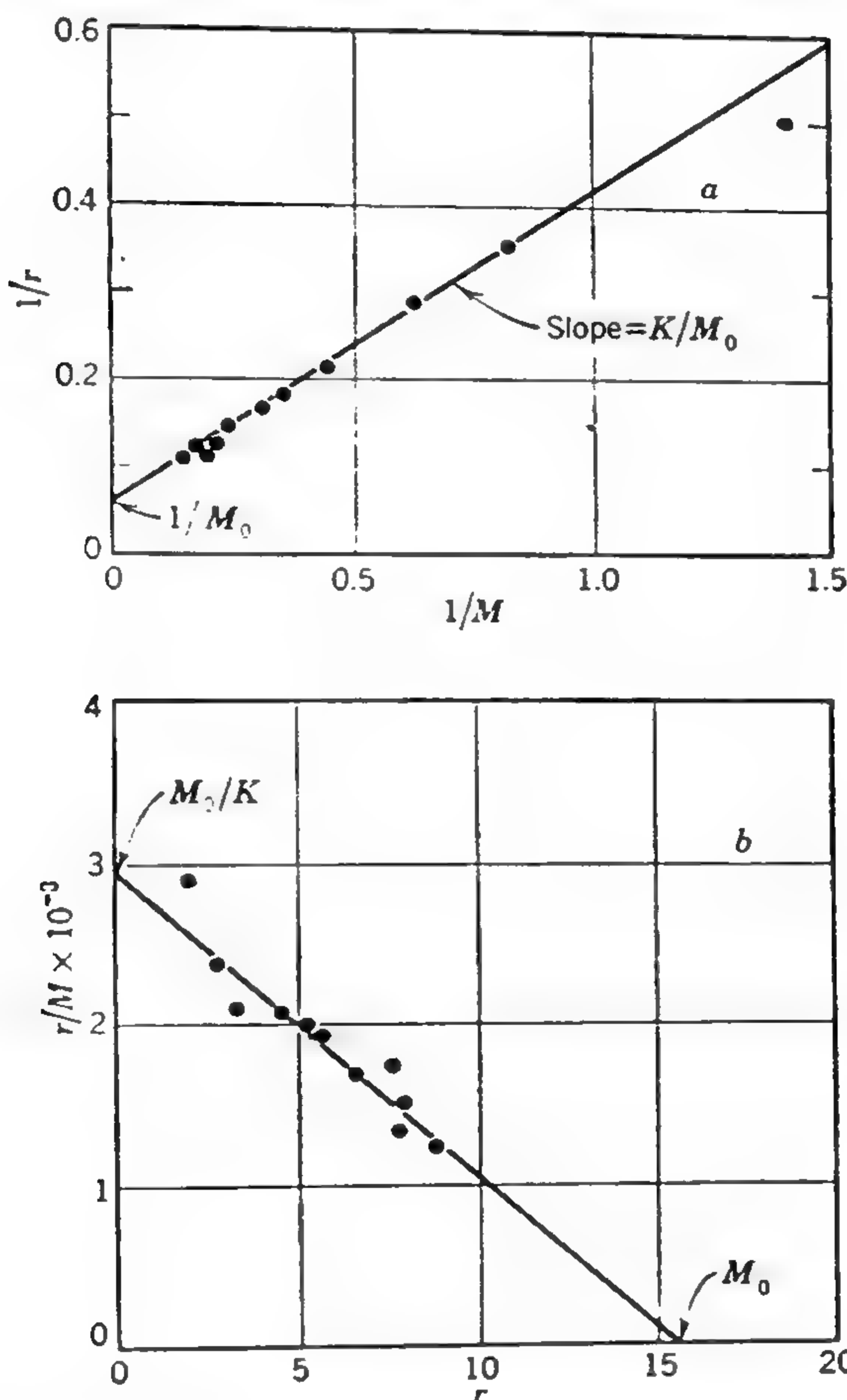


FIG. 4. Types of extrapolation to determine maximum number of calcium ions bound by casein (a) plotted according to equation 57 (b) plotted according to equation 59. (Klotz.)

Four experimental methods have been used to measure the extent of ion binding by proteins: (1) We have noted in Chapter 7 that the extent of hydrogen binding by a protein is determined electrometrically. There is no reason why electrometric methods could not be used for other ions, providing that the appropriate half-cell be available. (2) It is possible to use dialysis. That is, one places the protein solution on one side of a membrane that is permeable to the ion being used but impermeable to the pro-



tein. The ion is added to the system, and, after equilibrium has been established, chemical analysis establishes the distribution of ions on the two sides of the membrane from which the adsorption of ions can be calculated provided that the Donnan equilibrium has been suppressed by the addition of sufficient electrolyte. (3) The shift of the absorption spectrum incidental to adsorption can be used to evaluate dye-ion adsorption, and 4 Smith and Briggs<sup>59</sup> and Alberty and Marvin<sup>60</sup> have shown how it is possible to employ electrophoresis for this purpose.

Finally, it is well to point out that there is a strong resemblance between the Langmuir treatment and the Michaelis-Menten formulation of enzyme kinetics.

### MULTILAYER ADSORPTION

The adsorption of water vapor on a porous solid such as silica gel or on solid protein or solid cellulose usually yields an S-shaped curve when the amount of water adsorbed is plotted against the aqueous vapor pressure. An example of such an adsorption curve is that for collagen shown in Fig. 5.

We have already discussed the free energy and heat changes associated with adsorption of water vapor; here we wish to discuss the mechanism of adsorption. The first steep portion of the adsorption curve (Fig. 5) is an adsorption in the Langmuir sense and represents the filling-up of a monomolecular layer of water molecules on the collagen surface. The second rise is a type of capillary condensation. We have already pointed out that the vapor pressure of a liquid is decreased on a concave surface (see equation 4). The theory of capillary condensation has not, however, been satisfactorily formulated for the adsorption of vapor on porous solids. Brunauer, Emmett, and Teller<sup>61</sup> have treated this problem in some detail and have proposed an interesting and stimulating theory of such S-shaped adsorption curves. Essentially, they generalized Langmuir's adsorption theory to include multilayer adsorption. They considered the rates of evaporation and of condensation, not only of a unimolecular adsorbed layer but also of  $n$  such layers. They attribute the heat of adsorption of all but the first adsorbed layer to the heat of liquefaction of the vapor. The author is not prepared to admit this simple interpretation of the heat of adsorption of the layers beyond the first layer; the situation is more complex than that. This interpretation, however, is not an essential part of their theory. All that has to be agreed upon is that a heat term is involved in the multilayer adsorption. From these considerations, they were able to derive the

<sup>59</sup> R. F. Smith and D. R. Briggs, *J. Phys. & Colloid Chem.* **54**, 33 (1950).

<sup>60</sup> R. A. Alberty and H. H. Marvin, Jr., *J. Phys. & Colloid Chem.* **54**, 48 (1950).

<sup>61</sup> S. Brunauer, P. H. Emmett, and E. Teller, *J. Am. Chem. Soc.* **60**, 309 (1938).

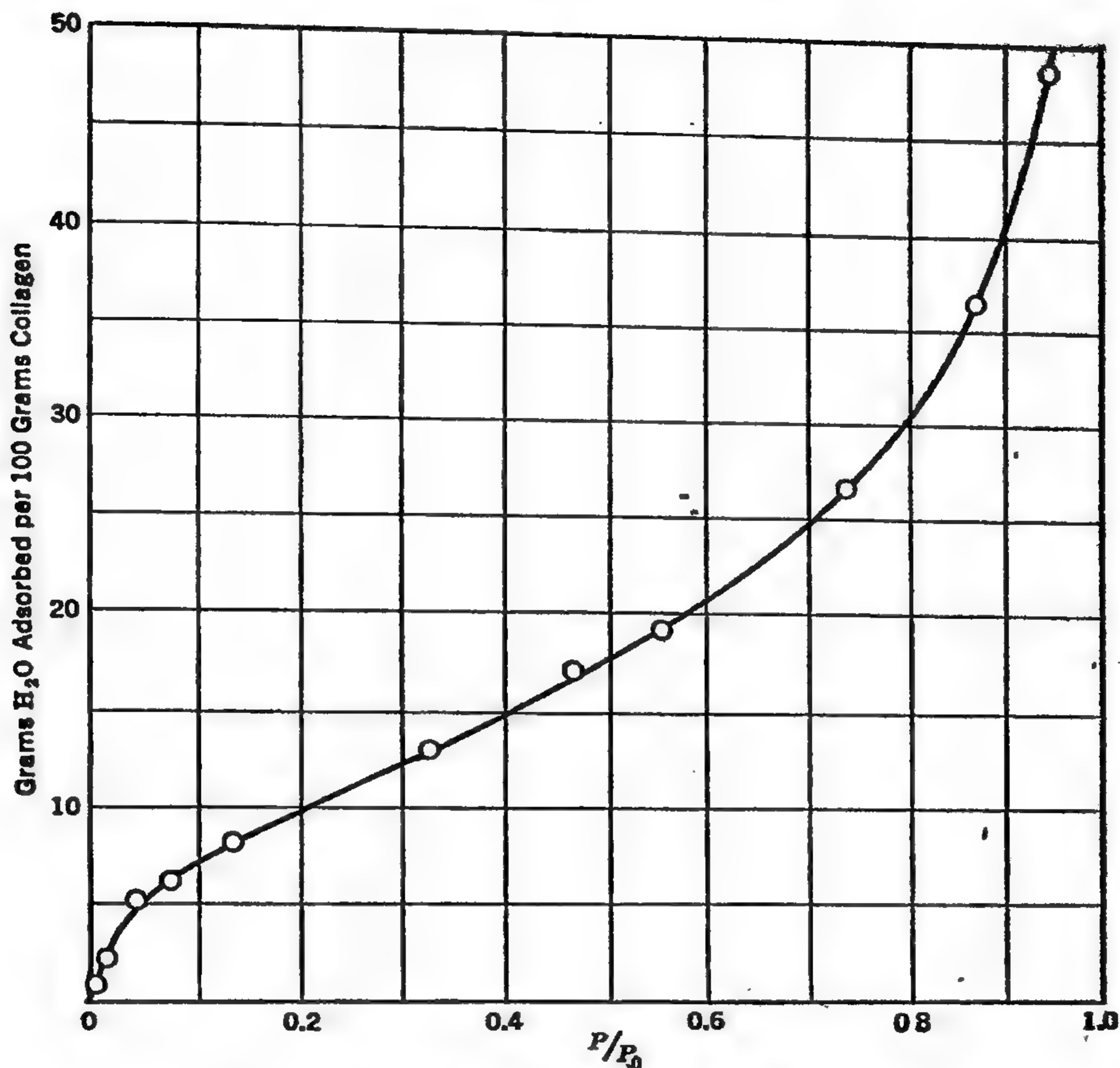


FIG. 5. Adsorption of water vapor on collagen at 25° C.

following equation to describe the adsorption of vapors on free surfaces.

$$V = \frac{V_m C P}{(P_0 - P)[1 + (C - 1)P/P_0]} \quad (61)$$

where  $V$  is the volume of adsorbed gas at the gas pressure  $P$ ,  $V_m$  is the volume of gas adsorbed when the entire adsorbent surface is covered with a complete unimolecular layer,  $P_0$  is the vapor pressure at saturation pressure of the gas,  $C$  is a constant which is very nearly given by the relation

$$C = e^{(H_1 - H_2)/RT} \quad (62)$$

where  $H_1$  is the heat of adsorption of the first unimolecular layer directly on the adsorbent and  $H_2$  is the heat of adsorption of other layers. Brunauer Emmett, and Teller believed  $H_2$  to be the heat of liquefaction of the gas

At low gas pressures equation 61 reduces to

$$V = \frac{V_m CP}{P_0[1 + (CP/P_0)]} \quad (63)$$

which, as will be recognized, is a form of the Langmuir equation. For the purpose of evaluating constants and testing equation 61, it is thrown into the form

$$\frac{P}{V(P_0 - P)} = \frac{1}{V_m C} + \frac{C - 1}{V_m C} \cdot \frac{P}{P_0} \quad (64)$$

A plot of  $P/[V(P_0 - P)]$  against  $P/P_0$  should give a straight line whose intercept is  $1/V_m C$  and whose slope is  $(C - 1)/V_m C$ . The constants  $V_m$  and  $C$  can thus be evaluated. As we have noted,  $V_m$  is the volume of adsorbate required to form a complete unimolecular adsorbed layer. The constant  $V_m$  thus becomes a measure of the surface area of the solid. Emmett<sup>22</sup> discusses the use of  $V_m$  to evaluate the surface area of fine powders. In order to do this we must be able to assign the proper dimensions to the adsorbed molecule. There is some ambiguity in this assignment. Do the adsorbed molecules exist at the surface as gases, liquids, or solids? He proposes the equation

$$\text{Area per molecule} = 1.585 \times 10^{-6} \left( \frac{M}{\rho} \right)^{1/3} \quad (65)$$

where  $M$  is the molecular weight of the gas, and  $\rho$  is the density of the solidified or liquefied gas. This equation is derived on the assumption that the molecules are hexagonally close packed in the solidified and in the liquefied gas.

If the thickness of the adsorbed film cannot exceed some finite number,  $n$ , owing to the dimensions of the capillaries, then we have instead of equation 61 the following extended equation

$$V = \frac{V_m CP/P_0}{(1 - P/P_0)} \left( \frac{1 - (n + 1)(P/P_0)^n + n(P/P_0)^{n+1}}{1 + (C - 1)P/P_0 - C(P/P_0)^{n+1}} \right) \quad (66)$$

In order to evaluate the constants in equation 66, we plot as before  $P/[V(P_0 - P)]$  against  $P/P_0$ . We use the linear portion of the curve to find the constants  $C$  and  $V_m$ . These two constants along with the experimental data are substituted in equation 66, and the best average value for  $n$  is determined. Figure 6 shows the plot of the data on collagen given in Fig. 5 according to the method of Brunauer et al.

<sup>22</sup> P. H. Emmett, *Advances in Colloid Science*, Interscience Publishers, Inc., New York, 1942.



If we assign the value of 10.9 square Å for the area occupied by one water molecule on the surface, we can calculate the surface area of the collagen. One cubic centimeter of water covers an area of  $\frac{10.9 \times 6.023 \times 10^{23}}{18 \times 10^{20}}$  or  $0.36 \times 10^4$  square meters of surface. From Fig. 6 the value of  $V_m$  is 9.53 cc. per 100 grams of collagen. Accordingly, the surface area of 100 grams of collagen is  $9.53 \times 0.364 \times 10^4$  or  $3.48 \times 10^4$  square meters.

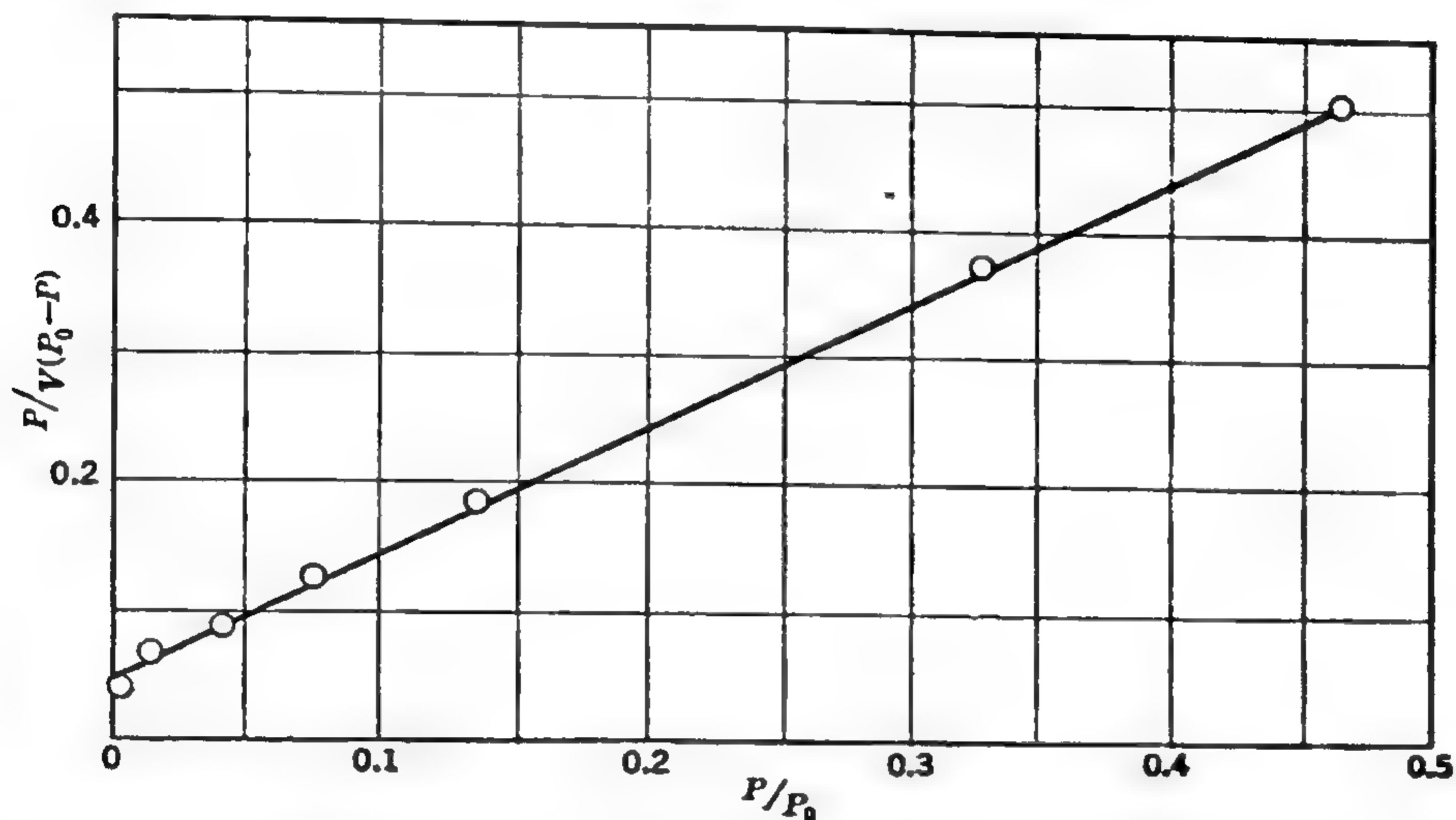


FIG. 6. The adsorption data of water on collagen at 25° C. plotted to obtain the constants  $V_m$  and  $C$ .

Table 4 gives the B.E.T. constants  $C$  for a series of proteins as well as  $a_1$  which is the number of grams of water required to complete the first layer of adsorbed water and  $a_s$  which is the number of grams of water required to saturate the solid protein.

Pauling<sup>63</sup> has preferred to interpret the first layer of adsorbed water on the protein ( $a_1$ ) as a measure of the number of hydrophilic groups available for water binding rather than as representing a completion of a condensed monolayer of water molecules.

Sponsler, Bath, and Ellis<sup>64</sup> have pointed out that there are two main types of hydrophilic groups in proteins: the polar side-chains groups of amino acid residues such as glutamic acid, arginine, tyrosine, etc., and the carbonyl and imino groups of the peptide bond. In short, these are the groups that are capable of forming hydrogen bonds. As Pauling notes, however, the peptide carbonyl and the imino groups probably cannot par-

<sup>63</sup> L. Pauling, *J. Am. Chem. Soc.* 67, 555 (1945).

<sup>64</sup> O. L. Sponsler, J. D. Bath, and J. W. Ellis, *J. Phys. Chem.* 44, 996 (1940).

ticipate to any great extent in water binding because they are already saturated by groups in the protein or because of steric hindrance. Making the assumption that the polar side-chain residues are the principal water binders, Pauling was able to show a fairly close relation between the number of water molecules adsorbed in the first layer and the number of polar groups.

Mellon, Korn, and Hoover<sup>65</sup> have compared the water adsorption of casein and of benzoylated casein (blocking of amino groups) and have concluded that the free amino group in the amino acid residue side chains play an important role in the adsorption of water vapors. Later these workers<sup>66,67</sup> considered the contribution of the peptide bond to water adsorption and also the influence of the physical state of the protein. They concluded that the protein molecules can be subject to drastic treatment without appreciably influencing the water-binding capacity.

Actually, it is possible to make a B.E.T. plot for concentrated sulfuric acid and obtain a good linear relation where the amount of water adsorbed is considered to be the water associated with 100 grams of dry acid and the corresponding aqueous vapor pressure is used. At 25° C. such a plot indicates that 3.87 moles of water are bound per mole of acid for the first layer. From structural considerations sulfuric acid should be able to bind 4 water molecules. A hydrophilic group in a protein would be expected to suffer steric hindrance to water binding much more than sulfuric acid molecules.

The theoretical treatment of multilayer adsorption has accumulated an extensive literature.

## CHROMATOGRAPHY

Adsorption has numerous laboratory applications, one of the more important of which is chromatographic analysis. The technique, in principle, is a simple one. A suitable adsorbent is packed in a vertical glass tube and a solution of the substance that it is desired to separate into its various components is poured into the tube. The component with the greatest affinity for the adsorbent is adsorbed first; the component with the next greatest affinity is adsorbed next; and so on. There is, however, at this stage, considerable overlapping of the components in the column. The chromatogram is now developed by passing some of the pure solvent into the column, whereupon each component of the mixture dissolves slightly as the solvent passes through the column but is readsorbed lower down. The net effect is to remove the less readily adsorbed material from the upper layer and carry it farther down the column. Under favorable circumstances the

<sup>65</sup> E. F. Mellon, A. H. Korn, and S. R. Hoover, *J. Am. Chem. Soc.* **69**, 827 (1947)

<sup>66</sup> E. F. Mellon, et al. *J. Am. Chem. Soc.* **70**, 3040 (1948).

<sup>67</sup> E. F. Mellon, A. H. Korn, and S. R. Hoover, *J. Am. Chem. Soc.* **71**, 2761 (1949)



columns will show a separate band for each component and if the substances are colored a separate band for each component will be clearly visible.

The botanist Tswett<sup>66</sup> first described the technique of chromatography and used it to separate plant pigments. Since that time chromatography has assumed major importance for the purification and analysis of biochemicals. In the short space at our disposal it would be unwise to attempt even a cursory survey of chromatography. It is, however, fruitful to outline the basic principles.

The technique of chromatography is closely related to that of counter-current distribution which Craig and associates<sup>67</sup> have made into an effective tool for purification; a short consideration of countercurrent distribution will aid in understanding chromatography. In effect, the counter-current-distribution apparatus is a series of separatory funnels. Suppose we place in the first of a series of separatory funnels a given volume of a liquid saturated with another, heavier liquid in which it is not completely miscible. A given weight of a solute is added and dissolved. The other funnels of the series contain equal volumes of the lighter liquid saturated with the denser liquid but containing no solute. A given volume of the heavier liquid saturated with the less dense liquid is now added to the first funnel and shaken. The bottom liquid phase is then transferred to the second funnel and shaken. The liquid removed from the first funnel is replaced by an equal volume of the heavier liquid. Successive transfers are made, the solute distributing itself between the two liquid phases. If the volume of the lighter liquid in each funnel be denoted by  $V_0$  and the volume of the heavier liquid by  $V_1$ , we have

$$\alpha = \frac{W_0 V_1}{W_1 V_0} \quad (67)$$

where  $\alpha$  is the partition coefficient of the solute and is equal to the ratio of the grams of solute per cubic centimeter in the upper liquid to the grams of solute per cubic centimeter in the lower liquid phase.  $W_0$  is the total weight of the solute in the upper liquid and  $W_1$  is the weight of the solute in the lower liquid. If we start the experiment with unit weight of solute such that the sum of  $W_0$  and  $W_1$  in the first funnel is unity, then the amount of solute transferred from the first to the second funnel is  $V_1 (V_1 + \alpha V_0)$  and the amount remaining is  $[1 - V_1 (V_1 + \alpha V_0)]$ . If we follow through the rather laborious calculations for each funnel after each new transfer, we can obtain the amount of solute in any funnel after any given number of transfers. Such calculations have been made by Martin and Synge<sup>68</sup> who

<sup>66</sup> M. Tswett, *Ber. deutsch. botan. Ges.* 24, 316 (1906).

<sup>67</sup> L. C. Craig, J. D. Gregory, and G. T. Barry, *Cold Spring Harbor Symposium Quant. Biol.* 14, 24 (1950).

<sup>68</sup> A. I. P. Martin and R. L. M. Synge, *Biochem. J.* 35, 1358 (1941).



concluded that the quantity of material in any funnel is a term in the binominal expansion and the quantity of solute in any funnel,  $r + 1$ , is

$$Q_{r+1} = n! \left(1 - \frac{V_1}{V_1 + \alpha V_0}\right)^{n-r} \left(\frac{V_1}{V_1 + \alpha V_0}\right)^r \quad (68)$$

where  $n$  is the total number of funnels in which transfers have been made.

Martin and Synge were able to show that, when  $n$  and  $r$  are large, the solute will distribute itself according to a Gaussian distribution curve and the funnel that has the maximum amount of solute in it will be

$$r = \frac{(V_1 + V_0)n}{V_1 + \alpha V_0} \quad (69)$$

At the beginning, all the solute is in the first funnel and the "distribution" of solute is as narrow as it can be. As the transfers are continued more and more funnels contain solute so that the distribution curve broadens as the transfers are continued. The ability to separate two or more solutes from each other will depend on the distribution coefficient  $\alpha$  and the volumes  $V_0$  and  $V_1$ . For example, the relative positions of the maximum concentrations of two solutes can be obtained from equation 69 and is

$$\frac{r_1}{r_2} = \frac{V_1 + \alpha_2 V_0}{V_1 + \alpha_1 V_0} \quad (70)$$

If  $V_0$  and  $V_1$  are equal, then the relative positions of the two maxima would be  $(1 + \alpha_2)/(1 + \alpha_1)$ .

Chromatography involves the same principles as does the countercurrent distribution. The immobile phase is the solid adsorbent, and the distribution takes place between the moving liquid and the surface of the solid adsorbent. There are a great many "transfer funnels" and, in general, it is possible to effect more complete separation than is true of countercurrent distribution.

The partition coefficient  $\alpha$  is not independent of the concentration of the solute, and at higher concentrations of solute this can lead to complications. This dependence of the adsorption coefficient in chromatography on the concentration of the solutes is even more pronounced and results in the advancing front of a band being sharp but renders the tail of the band diffuse and spread out.

The equation for the relative position of an adsorbed band in chromatography can be written

$$\frac{W}{V} = \frac{V_T}{\rho V_a \alpha + V_s} \quad (71)$$

where  $W$  is the volume rate of migration of a band expressed in cubic centi-

meters per second,  $V$  is the volume of the solvent passed into the column in cubic centimeters per second.  $V_T$  is the total volume enclosed in the tube,  $V_a$  is the volume of the adsorbent in the tube,  $V_s$  is the volume of the solvent in the tube,  $\rho$  is the density of the adsorbent, and  $\alpha$  is the adsorption coefficient of the solute at the concentration in question, i.e., the ratio between the amount of solute adsorbed per gram of dry column material and the concentration of the solute in solution.

If the substances to be separated have distinctive colors, then the separation and identification of the bands are greatly facilitated. However, if the substance possesses no such colors, the identification is obviously complicated. Very ingenious schemes have been devised to circumvent these difficulties.

Tiselius and Claesson<sup>71</sup> have developed a continuous reading refractometric method which enables them to analyze the solution leaving the chromatography column.

Moore and Stein<sup>72</sup> have described an automatic fraction collector for elution chromatography which is very effective.

Possibly the most ingenious device is that of filter paper chromatography, first suggested by Consden, Gordon, and Martin,<sup>73</sup> in which a small amount of a mixture of the substances to be separated is deposited near one end of a strip of filter paper and the tip of this end of the paper is dipped in a proper organic solvent, such as an 80 per cent aqueous phenol solution. The phenol flows along the filter paper strip by capillarity, and the substances in the mixture move along the strip as bands. The distance that the substances move is characteristic and is expressed as the relative flow ( $R_F$ ). The relative flow is the ratio of the distance traveled by the substance to the distance of flow of the solvent. The position of the bands on the filter paper is obtained by spraying the paper with some material capable of forming a colored product with the material on the filter paper.

#### ADSORPTION AND CHEMICAL BINDING

For many years the cause of adsorption at solid-gas or at solid-liquid surfaces was clouded in mystery. People spoke of adsorption as though it were completely different from chemical binding. It is now believed that there is no fundamental difference between adsorption and chemical binding; identical types of forces are operating. The apparent lack of a stoichiometric relation in adsorption reactions is due to the varying affinities of the adsorbent molecules for the adsorbate molecules. The adsorbent molecules

<sup>71</sup> A. Tiselius, *Advances in Colloid Science*, Interscience Publishers, Inc., New York, 1942; *Advances in Protein Chem.* 3, 67 (1947).

<sup>72</sup> S. Moore and W. H. Stein, *J. Biol. Chem.* 178, 53 (1949).

<sup>73</sup> R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.* 38, 224 (1944).



immediately on the surface are more or less completely exposed, as, for example, the molecules on the sharp edges of the molecular hills of the surface. Other molecules on the surface are less exposed. This results in a graded series of affinities which leads to the typical parabolic relation between concentration and the amount adsorbed. In general, it is believed that adsorptive forces extend only about one molecular distance away from the surface, so that it is to be anticipated that, unless capillary condensation takes place, the adsorbed film will be unimolecular at saturation of the surface.

Physical chemists find it convenient to distinguish two types of adsorption. The physical or van der Waals adsorption is characterized by a low heat of adsorption, whereas activated or chemisorption has a much larger heat of adsorption. The distinction is of value in discussing the adsorption of gases on solids.

#### SPREAD MONOLAYERS

When a small amount of an insoluble substance is placed on a clean water surface, one of two things may happen. The substance may remain as a compact mass, leaving the rest of the surface clean, or it may spread over the surface to form a very thin film.

The first experiments on spreading were made many years ago. In 1890 Lord Rayleigh<sup>74</sup> spread olive oil on water and measured the thickness of the spread film. He found it to be about  $16 \text{ \AA}$  thick.

Pockels<sup>75</sup> discovered that spread surface films could be pushed along with barriers and that such films could be compressed by moving the barriers together. She also measured the surface tension as a function of the area of the film and found that, at complete expansion, the surface tension was independent of the area of the film, but below a certain critical area the surface tension dropped rapidly as the area was decreased.

Langmuir<sup>76</sup> introduced new techniques and interpretations into this field of investigation. He used for the first time a floating mica strip to register the film "pressure." The fatty acids with which Langmuir worked were applied to the surface by first dissolving them in a volatile solvent such as benzene or petroleum ether and dropping the solution on the clean water surface. The petroleum ether evaporated and left the pure fatty acids spread on the surface. Langmuir measured the area occupied by a given weight of straight-chain fatty acids, and, knowing the number of fatty acid molecules present, he was thus able to calculate the area per fatty acid molecule.

<sup>74</sup> Lord Rayleigh, *Proc. Roy. Soc. (London)* **47**, 364 (1890).

<sup>75</sup> A. Pockels, *Nature* **43**, 437 (1891).

<sup>76</sup> I. Langmuir, *J. Am. Chem. Soc.* **39**, 1848 (1917).



The fact that the fatty acid molecules contained the hydrophilic  $\text{—COOH}$  group suggested that in compressed films the molecules were oriented with these groups sticking into the water and that the hydrocarbon chain pointed into the air. Langmuir further suggested that in the uncompressed film the molecules lie flat on the surface. The films of fatty acids containing less than 14 carbon atoms per molecule dissolved when compressed. Evidently the buoyancy of the hydrocarbon chain was not sufficient to overcome the attraction of the  $\text{—COOH}$  group for water.

Suggestions that molecules were oriented at air-water surfaces were made previous<sup>77</sup> to and simultaneous<sup>78</sup> with Langmuir's publication, but Langmuir's demonstration of molecular orientation at a surface was so clear cut and beautiful that credit is usually given him for the discovery.

There are several properties of spread films which can be studied conveniently and these are: (1) force-area curves, (2) film potential area measurements, (3) film viscosities, and (4) optical appearance of the film with a dark field illumination.

Force-area measurements have been most frequently studied and indeed were the kind of measurement that Langmuir first employed. It is important to realize that the so-called film pressure is the difference in surface tension between that of the clean surface and that of the surface covered with the spread film. Langmuir accomplished such measurement by separating the surface of the solution into two parts by means of a mica float. One part was covered with the surface film which could be compressed; the other part of the surface contained no spread film. The clean surface exerted a greater tension on the mica float than that containing the spread film. By adding counterweights to bring the mica float back to its equilibrium position, it was possible to measure this difference in surface tension directly. Puddington<sup>79</sup> has described a two-dimensional Bourdon gauge which can be used to measure film pressures.

Another method of measuring the film pressure is to determine the surface tension of the clean surface and also the surface tension in the presence of the film. The most convenient way of making these measurements is by means of the Wilhelmy balance.

The usual procedure is to add to the surface a certain amount of spreadable material and then compress the spread film with a movable barrier and measure the corresponding film pressure by the methods described above. Fatty acid films on aqueous solutions have been explored in some

<sup>77</sup> W. B. Hardy, *Proc. Roy. Soc. (London)* **86A**, 610 (1912); **88A**, 303 (1913).

<sup>78</sup> W. D. Harkins, *J. Am. Chem. Soc.* **39**, 354 (1917); **39**, 541 (1917).

<sup>79</sup> I. E. Puddington, *J. Colloid Sci.* **1**, 505 (1946).

detail, and it has been shown that such films can exist in a number of states which are:<sup>20</sup>

1. Condensed film, in which the molecules are closely packed and steeply oriented to the surface.
2. Liquid-expanded films, which are still coherent but occupy a much larger area than condensed films. These films can form on the surface a separate phase, a gaseous film which is in equilibrium with the liquid phase.
3. Vapor-expanded films, which are similar to liquid-expanded film but have less cohesion and do not show a region of constant surface pressure.
4. Gaseous or vapor films, in which the molecules are separate and show independent motion. Adsorbed films of soluble, capillary-active substances are nearly always gaseous.

We see that there is a close analogy between phase transitions in two dimensions (surface films) and phase transitions in three dimensions and a single substance, under the proper conditions of temperature and surface pressure, may exist in several states.

### GASEOUS FILMS

The term "gaseous film" is an unfortunate one. Such films are not in any sense of the word two-dimensional gases but exist rather as surface solutions. The term has become so firmly established in the literature, however, that it appears unwise to substitute a less ambiguous one.

To expand a gaseous spread film, water must be transferred from the bulk phase to the surface phase. If  $a_0$  be the activity of the water underlying the surface,  $a$  the activity of water in the clean surface, then, to expand the surface by transferring one mole of water from bulk into the surface at constant temperature and pressure, the free energy required is evidently

$$\Delta F = A_1 \sigma_0 = RT \ln \frac{a}{a_0} \quad (72)$$

where  $A_1$  is the area occupied by one mole of water,  $\sigma_0$  is the surface tension of water,  $R$  is the gas constant, and  $T$  is the absolute temperature. The corresponding free-energy change for a surface containing  $n_1$  moles of spread substance is

$$\Delta F_1 = A_1 \sigma_1 = RT \ln \frac{a_1}{a_0} \quad (73)$$

where  $a_1$  is the activity of the water in the surface containing the spread

<sup>20</sup> N. K. Adam, *The Physics and Chemistry of Surfaces*, third edition, Oxford University Press, London, 1941.



material. Subtracting equation 73 from equation 72, we have

$$A_1(\sigma_0 - \sigma_1) = FA_1 = RT \ln \frac{a}{a_1} \quad (74)$$

where  $F$  is the film pressure. Now  $a/a_1$  is nearly equal to  $N/N_1$  where  $N$  is the mole fraction of water in the clean surface and  $N_1$  is the mole fraction of water in the surface containing spread film.  $N$  is evidently unity and, accordingly, equation 74 becomes

$$FA_1 = -RT \ln \left( 1 - \frac{n_2}{n_1 + n_2} \right) \quad (75)$$

where  $n_1$  is the number of moles of water in the surface on which protein has spread. Since  $n_1$  is very much greater than  $n_2$ , we have, after expanding the logarithmic term and rearranging and neglecting higher terms of the expansion,

$$n_1 FA_1 = n_2 RT \quad (76)$$

Evidently,  $n_1 A_1$  is the area occupied by the water and

$$n_1 A_1 = A - n_2 S_P \quad (77)$$

where  $A$  is the total area of the surface and  $S_P$  is the area per mole of protein. Substituting equation 76 into equation 77, we have after rearranging

$$FA = n_2 RT + n_2 S_P F \quad (78)$$

If  $FA$  be plotted against  $F$ , the intercept is evidently  $n_2 RT$  and the slope of the line is  $n_2 S_P$ . When  $FA$  is expressed in dyne-centimeters per molecule then the product is equal to  $kT$  where  $k$  is Boltzmann's constant and  $T$  is the absolute temperature. At  $25^\circ \text{C}$ . this product is equal to  $411 \times 10^{-16}$  erg. If the area be expressed in  $(\text{\AA})^2$ , then  $kT$  per molecule is 411 at  $25^\circ \text{C}$ .  $S_P$  will then be expressed in  $(\text{\AA})^2$ . Frequently, the amount of material on the surface is expressed in square meters per milligram, and  $RT$  at  $25^\circ \text{C}$ . becomes  $411 \times 10^{-16} \times 6.02 \times 10^{23} / (1000 \times 10,000)$  which is 2478. A study of force-area relations of a gaseous film thus offers a means for the determination of the molecular weight of the spread substance. Evidently, one mole of the spread substance will yield an intercept of 2478 at zero pressure when  $FA$  is plotted against  $F$ . The actual intercept is obtained, and, accordingly, we know how many moles of the substance is in the film. Knowing also the weight of the substance applied to the film, we can calculate the molecular weight. The molecular weight is equal to 2478 divided by the intercept value of  $FA$  at zero value of  $F$ . Guastalla<sup>21</sup> was the first to calculate the molecular weight of proteins spread in surface films in the gaseous region.

<sup>21</sup> I. Guastalla, *Compt. rend.* 208, 973 (1939).



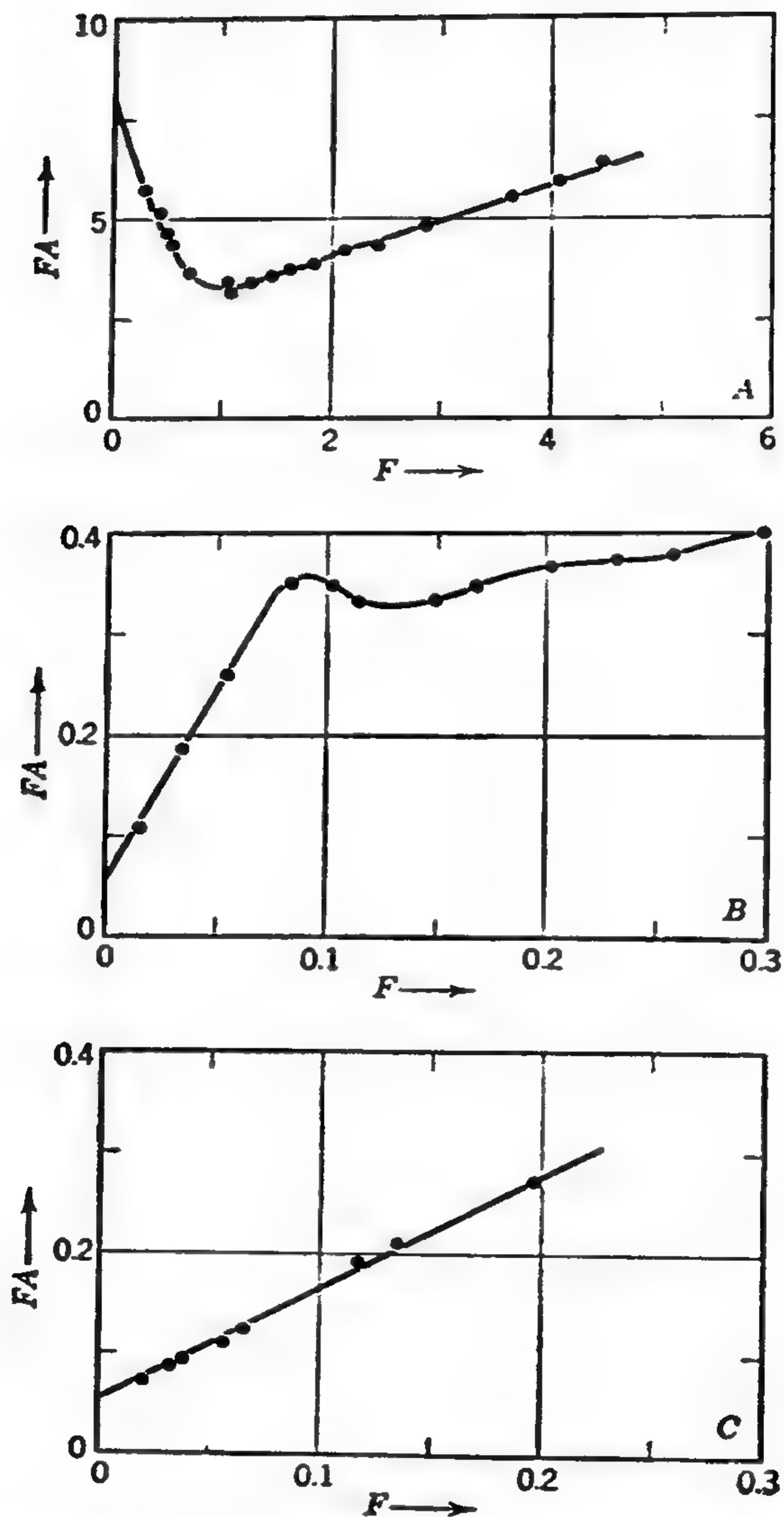


FIG. 7.  $FA$  vs.  $F$  plots in low-pressure region. Area expressed in square meters per milligram and pressure in dynes per centimeter. (A) Sodium lauryl sulfate spread on 19 per cent ammonium sulfate. (B) Insulin spread on 5 per cent ammonium sulfate containing 2 per cent glycerol. (C) Pepsin spread on 5 per cent ammonium sulfate.

Figure 7 shows three typical  $FA$  vs.  $F$  plots for gaseous films. Figure 7A is that given by sodium lauryl sulfate.<sup>82</sup> Figure 7B shows an  $FA$  vs.  $F$  plot for insulin spread on 5 per cent ammonium sulfate containing 2 per cent glycerol,<sup>83</sup> and Fig. 7C shows the results with pepsin spread on a 5 per cent ammonium sulfate solution.

The lauryl sulfate exhibits a minimum which is attributed to the formation of micelle on the surface as the film is compressed. The micelle contain, on the average, 4 lauryl sulfate molecules. Other substances exhibit such minima. For example, dibasic esters of fatty acids having carboxyl groups at each end show minima as the films are compressed.<sup>84</sup>

The break in the  $FA$  vs.  $F$  curve for insulin film is attributed to a compression of the expanded gaseous molecules themselves with increasing film pressure. The area of the expanded gaseous molecules is about 3.3 square meters per milligram, whereas the compact form of the gaseous molecules is about one square meter per milligram.

The advantages of the spread-film technique for the determination of the molecular weight are that the apparatus is inexpensive, the time required for the measurement is short, and the amount of material required is very small (20 to 30 micrograms are usually sufficient).

We see from the simple derivation outlined above leading to equation 78 that the pressure exerted by a spread film is closely analogous to an osmotic pressure. According to this derivation,  $n_2Sp$  is a direct measure of the surface occupied by the spread substance, and, indeed, experiments tend to confirm this conclusion: the gaseous areas calculated for a number of proteins are very nearly equal to those obtained by full compression of the film to the beginning of the collapse pressure. There is something unclear about this situation, however, because according to modern theory of osmotic pressure the solute molecules should exhibit a co-volume in the same sense that the constant  $b$  in the van der Waals gas equation represents a co-volume and involves a collision diameter; the co-volume for spherical molecules is four times the actual volume. For rod-shaped and disk-shaped molecules, the co-volume is larger, and, for extreme molecular asymmetry, the co-volume increases very greatly.

#### PROTEIN FILMS AT HIGHER PRESSURES

An expanded spread film of protein can be freely blown about on the surface by a gentle current of air, as evidenced by the fact that, when talc is sprinkled on such an uncompressed film, the talc particles will readily respond to a gentle air current and after the air pressure is stopped the talc

<sup>82</sup> H. B. Bull, unpublished data.

<sup>83</sup> H. B. Bull, *J. Biol. Chem.* **185**, 27 (1950).

<sup>84</sup> Adams and Jessup, *Proc. Roy. Soc. (London)* **A112**, 376 (1926).

particles continue to move, owing to their momentum. As the film pressure is increased on the spread film the talc moves with less freedom, until a pressure is finally reached at which the spread film becomes fairly rigid, as evidenced by the fact that, if the talc is displaced a small distance on the surface, it will return to its former position, owing to the elastic structure of the film and the film is said to have gelled. A protein film undergoes no true phase changes, as do the fatty acid films, but is gradually transformed with increasing film pressure from a gas at large areas and small pressures to a gelled film at small areas and high pressures.

The pressure region extending from about one dyne up to about 20 dynes per centimeter pressure has been studied repeatedly, and many force-area curves for this pressure region have been reported. A review of this work has been published.<sup>55</sup> The similarities between those force-area curves for the various proteins are much more striking than their differences. The resemblance is not to be wondered at; this is the region in which the spread protein molecules are packed together. Since all proteins are made up of amino acids connected by peptide bonds and since the average molecular weights of these amino acids show no large variations from protein to protein, it is to be expected that the amino acid residues of the various proteins would pack in a monolayer of peptide chains at high film pressures to substantially the same area. Figure 8 shows the force-area curve for egg albumin spread on 35 per cent ammonium sulfate.

The coefficient of compressibility offers a convenient characteristic of protein film in the high-pressure region. The coefficient of compressibility is

$$\delta = - \frac{dA}{A dF} \quad (79)$$

where  $A$  is the area of the film in square meters per milligram and  $F$  is in dynes per centimeter. When the coefficient of compressibility is plotted against the area of the film a well-defined minimum is usually observed in this plot which allows the area and pressure at minimum compressibility to be assigned without ambiguity. It is probable that the point of minimum compressibility of a protein film corresponds to the smallest area to which a film can be compressed without partial collapse of the film occurring.

Zocher and Stiebel<sup>56</sup> devised a dark-field ultramicroscope with which a spread film can be observed as it is compressed. This arrangement has been modified by Adam.<sup>57</sup> Inhomogeneities in the spread film are easily detected by this method, since light is not scattered by a homogeneous monolayer, whereas any unspread material or aggregates formed incidental

<sup>55</sup> H. B. Bull, *Advances in Protein Chem.* 3, 95 (1947).

<sup>56</sup> H. Zocher and F. Stiebel, *Z. physik. Chem.* 147, 401 (1930).

<sup>57</sup> N. K. Adam, *Trans. Faraday Soc.* 29, 90 (1933).



to collapsing of the film show up as brilliantly illuminated regions. It is observed that evidence of collapse of protein film appears at a film pressure that corresponds rather well to that of the point of minimum compressibility.

Calculations show that the areas corresponding to point of minimum compressibility of a series of proteins is very nearly 0.80 square meter per

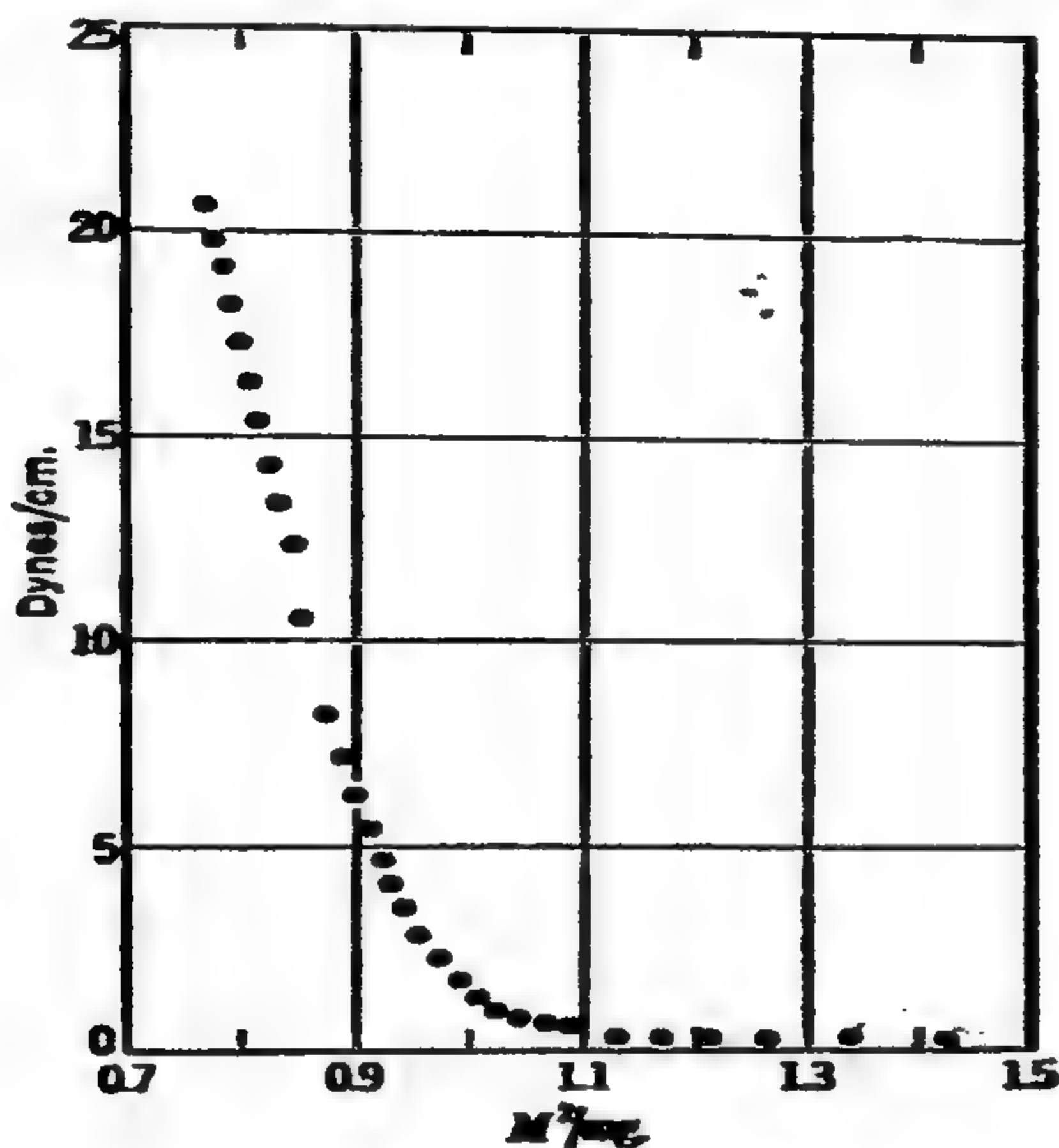


FIG. 8. Force-area curve of egg albumin spread on 35 per cent ammonium sulfate.

milligram. This value agrees well with the dimension of compact layer of peptide chains of average composition obtained from X-ray studies of proteins in bulk.

The actual structure of spread monolayers of proteins is about as obscure as the molecular structure of proteins themselves. It is one of the remarkable facts of nature that, when water-soluble, highly organized, native protein molecules are placed on an aqueous surface, they promptly spread on the surface to form insoluble films whose thickness, when compressed, corresponds to one peptide chain irrespective of the dimensions of the original, native molecule.

#### SURFACE POTENTIALS

Spread films can be characterized by surface potentials as well as by force-area measurements. In this type of measurement the change of the

electrical potential difference between the substrate solution and air produced by the compression of the spread film is studied. The experimental arrangement generally consists of a movable electrode above the surface of the film, a reversible electrode in the substrate solution, and the proper external circuit which permits the measurement of the potential difference between the air and the substrate solution. Descriptions of the apparatus have been given by Schulman and Rideal,<sup>30</sup> by Adam and Harding,<sup>31</sup> by Harkins,<sup>32</sup> by Philippi,<sup>33</sup> and by others.<sup>32, 33</sup>

If the electrical double layer at the surface be treated as a plane plate condenser, we have

$$\Delta r = \frac{4\pi d\sigma}{D} \quad (80)$$

where  $\Delta r$  is the difference between the potential of the clean surface and the potential after the monolayer is spread,  $d$  is the thickness of the double layer,  $\sigma$  is the electrostatic charge per square centimeter of surface, and  $D$  is the dielectric constant in the double layer. If  $\mu$  is the average dipole moment of the molecules of the spread film and  $\theta$  be the angle of tilt of this dipole moment from a normal to the surface, then

$$\Delta r = \frac{4\pi n\mu \cos \theta}{D} \quad (81)$$

where  $n$  is now the number of molecules per square centimeter. Equation 81 is found to be consistent with experiments in the low-pressure region on protein films, but for pressures above about one dyne per centimeter it leads to very questionable conclusions.

The measurement of film potential has value in two connections: (1) to detect inhomogeneities in the spread film and (2) to detect interaction of molecules injected under a spread film and the molecules of the film.

Schulman<sup>34</sup> reports that, if surface active molecules having hydrophilic heads and hydrophobic tails be injected under a protein film, three situations may arise. (1) If there is no association between the polar heads of the injected molecules and the polar groups in the film, no alteration in the film characteristics is noticed. (2) If there is association between the polar groups of the injected molecules and the film but no association between the

<sup>30</sup> J. H. Schulman and E. K. Rideal, *Proc. Roy. Soc. (London)* A130, 259 (1931).

<sup>31</sup> N. K. Adam and J. B. Harding, *Proc. Roy. Soc. (London)* A138, 411 (1932).

<sup>32</sup> W. D. Harkins, *J. Chem. Phys.* 1, 852 (1933); 3, 693 (1935).

<sup>33</sup> G. Th. Philippi, "On the Nature of Proteins," Thesis University of Leiden, 1936.

<sup>34</sup> E. F. Porter, *J. Am. Chem. Soc.* 59, 1583 (1937).

<sup>35</sup> A. A. Frost and V. R. Hurka, *J. Am. Chem. Soc.* 62, 3335 (1940).

<sup>36</sup> J. H. Schulman, *Trans. Faraday Soc.* 33, 1116 (1937).

hydrophobic tails and the film there is an absorption of the injected molecules under the film with consequent change in the surface potential but no increase in surface pressure. <sup>5</sup> If there is association between both the heads and tails of the injected molecules with the film, then a polar group of the film attracts a polar group of the injected molecules. The hydrophobic portion of the injected molecule associates with the hydrophobic portion of the film and thus penetration of the monolayer results. In this case the film pressure assumes a value that is an average one for the original monolayer and the injected molecule.

Film penetration can be completely prevented by compressing the film prior to injection to the equivalent pressure at which displacement of the penetrating substance would start. Film penetration although of great interest is very difficult to quantify because of the unknown amount of the injected material that remains in solution in the subphase.

Mason and Schindler <sup>6</sup> have used a modified film balance which permits automatic compensation for the pressure due to the penetration of the film by the solute.

In a mixed film containing more than one kind of surface-active molecule, an increase in film pressure will usually lead to displacement of one of the components from the film. It has been found <sup>7</sup> possible to make an estimation of the molecular-weight distribution of a mixture of peptides resulting from the partial hydrolysis of proteins on this basis. The partial hydrolysis is stopped and its average molecular weight measured by extrapolating to  $\pi = 0$  vs.  $\pi$  plot at zero pressure; the slope of this line gives a measure of the amount of material in the film. The film is then compressed to higher and higher pressures and held at these successive pressures for 5 minutes. After each compression the film is expanded and the average molecular weight determined. The molecular weight and the amount of material stretched out at each pressure can be calculated and accordingly, the molecular weight distribution of the peptides can be estimated.

### VISCOSITY OF FILMS

The viscosity of surface films has been measured in one of two ways. The passage of the film through a small channel can be observed <sup>8, 9</sup> or the resistance of a disk in the surface can be measured <sup>10, 11, 12, 13</sup>

- <sup>5</sup> R. Mason and J. E. Schindler, *J. Colloid Sci.* **4**, 30 (1949).
- <sup>6</sup> R. E. Ball and J. W. Egan, *J. Am. Chem. Soc.* **70**, 1147 (1948).
- <sup>7</sup> M. J. Cantow and J. E. Schindler, *J. Colloid Sci.* **18**, 10 (1963).
- <sup>8</sup> W. J. Barnes and J. E. Kitchener, *J. Chem. Phys.* **4**, 55 (1936).
- <sup>9</sup> J. E. Schindler, *Adv. Spring Forum Symposium*, *Chem. Res.* **4**, 177 (1963).
- <sup>10</sup> M. J. Cantow, *J. Chem. Phys.* **35**, 285 (1961).
- <sup>11</sup> J. E. Schindler, *J. Phys. Chem.* **43**, 987 (1939).
- <sup>12</sup> J. E. Schindler and V. J. Schindler, *Chem. Res.* **24**, 131 (1963).



The surface viscosity, in general, increases greatly as a surface film is compressed. This is particularly true of protein films. Joly<sup>103</sup> reports, for example, that below a certain critical film pressure the surface viscosity of protein film is Newtonian. Above a certain pressure the protein film gels. Between these pressures viscosity increases very greatly, and the flow is no longer Newtonian (rate of flow proportional stress). Joly calculates the energy of activation of flow and concludes that the unit of flow in the spread film of protein is much less than that corresponding to one molecule: the area of this unit is about  $90 (\text{\AA})^2$ .

### BUILT-UP MONOLAYERS

Blodgett<sup>104</sup> has described a technique by which successive monolayers of fatty acids may be built up on solid surfaces. The monomolecular film is transferred from the water surface to glass or metal slides by raising the slide slowly out of water upon which a monolayer has been spread. A fatty acid film is deposited on a calcium bicarbonate buffer. The pH of the calcium bicarbonate buffer is adjusted to the needs of the experiment by bubbling  $\text{CO}_2$  through the buffer. Two types of deposition are described by Blodgett. The Y-deposition is obtained on the up, as well as on the down, trip of the slide; the X-deposition takes place only on the down trip of the slide. The governing factor in the type of deposition is the pH of the buffer. The Y-deposition is obtained at pH 7 and lower, and X-deposition at pH 9 and above. During deposition, a constant pressure is maintained on the film by means of a "piston oil." The piston oil spreads with an approximately constant pressure. Table 5 lists some piston oils used for this purpose.

TABLE 5  
"PISTON OILS" AND THEIR FILM PRESSURE

Piston Oil	F in Dynes per Centimeter
Tricresyl phosphate	9.5
Rape-seed oil	10.5
Castor oil	16.5
Neat's-foot oil	19.0
Oleic acid	29.5

If the index of refraction of the glass slide differs sufficiently from that of the deposited film, beautiful interference colors may be produced. The color depends on the thickness of the film and on the difference of index of refraction between the glass and the deposited film. As the index of refraction of fatty acids is about 1.4 and that of ordinary glass is also about 1.4,

<sup>103</sup> M. Joly, *Biochim. et Biophys. Acta* 2, 624 (1948).

<sup>104</sup> K. B. Blodgett, *J. Chem. Am. Soc.* 56, 495 (1934); 57, 1007 (1935).

no color results from films deposited on ordinary glass. Fatty acid films deposited on slides of optical glass having an index of refraction of 1.64 give beautiful and intense colors. The best colors, however, are exhibited by films deposited on polished metal slides such as those of chromium.

Y-films which are deposited on the up as well as down trip should have the methyl groups adjacent to methyl and carboxyl groups adjacent to carboxyls. X-films, on the other hand, should have the methyls of one layer adjacent to the carboxyls in the next layer. Actually, X-ray studies show <sup>105</sup> the X- and Y-films to have the same crystal structure.

Bikermann <sup>106</sup> has suggested that the deposited film ceases to be a monolayer and becomes aggregates or microcrystals when the film is deposited on a solid from a liquid surface. He came to this conclusion through deposition ratio studies. The deposition ratio is the ratio of the area of the spread film on the liquid to its area when deposited.

Epstein <sup>107</sup> has examined deposited monolayers and multilayers of normal fatty acids with the electron microscope and with electron diffraction. He concludes that the deposited fatty acids are grouped into two dimensional micelles, about 100 Å in diameter. The fatty acids in the center of a micelle are oriented normal to the surface of the substrate, and those around the perimeter of the micelle are tilted towards the center. As the micelle grows, the angle of tilt becomes greater because the fatty acids are close packed and the cross-sectional area of the hydrocarbon tails is 18.4 sq. Å, whereas that of the carboxyl group is 20.4 sq. Å. Eventually, there is insufficient cohesion to stabilize the outer fatty acids, and the micelle stops its growth when its diameter is about 100 Å.

#### DEPOSITED PROTEIN FILMS

Langmuir and Schaefer, <sup>108</sup> using essentially the same technique as described above for the deposition of fatty acid films, have deposited protein monolayers on solid surfaces. The slide must be conditioned with a layer of calcium or barium stearate before deposition of the protein; otherwise the protein film deposited on the up trip out of the surface will peel off on the down trip through the surface.

Astbury, Bell, Gorter, and Van Ormondt <sup>109</sup> have deposited up to seventeen hundred successive layers of egg albumin on chromium-plated metal slides. These films have a tendency to tear parallel to the direction in which the slide was moved through the liquid surface. The films were

<sup>105</sup> C. Holley, *Phys. Rev.* **53**, 534 (1938).

<sup>106</sup> J. J. Bikermann, *Proc. Roy. Soc. (London)* **170A**, 130 (1939).

<sup>107</sup> H. T. Epstein, *J. Phys. & Colloid Chem.* **54**, 1053 (1950).

<sup>108</sup> I. Langmuir and V. Schaefer, *Chem. Revs.* **24**, 181 (1939).

<sup>109</sup> W. T. Astbury, F. O. Bell, E. Gorter, and J. Van Ormondt, *Nature* **142**, 33 (1938).



birefringent (transmitted polarized light) when viewed perpendicular to the surface, the slow vibration being parallel to the direction of movement of the slide. The thickness of these films, measured by direct mechanical means, was 9.5 Å per monolayer, which is in excellent agreement with the results obtained with the surface balance. Unfortunately, no deposition ratios for the films are reported, and without this information a comparison with the thickness of the film on the liquid surface is really not justified.

Langmuir and Schaefer have deposited what they call S-films of proteins on solids. These protein films are produced by placing a drop of protein solution on a wet slide upon which had previously been deposited a stearic acid film conditioned with aluminum chloride or thorium nitrate. The protein solution, after being placed on such a slide, is washed off, the S-film remains on the slide.

Harkins, Fourt, and Fourt<sup>119</sup> have reported a very striking example of specific adsorption. An S-film of beef catalase was placed on a chromium-plated slide, and the slide exposed to a serum-containing beef anticatalase from rabbits. The anticatalase deposited on the slide. It was then possible to deposit another layer of catalase. Successive alternating layers of catalase and anticatalase were thus built up. If the catalase film was exposed to serum not containing anticatalase, little or no protein was taken up by the slide. Incidentally, the deposited catalase retained its ability to decompose  $H_2O_2$  even when it was covered by a layer of anticatalase.

### MEASUREMENT OF THICKNESS OF DEPOSITED FILMS

In order to make quantitative experiments on deposited films, it is necessary to be able to measure their thickness. Blodgett and Langmuir<sup>120</sup> accomplish this by a study of the interference produced by reflection of monochromatic light from the surface of the deposited film and from the surface of the metal slide. A more precise method is that of Rothen<sup>121</sup> who has devised an instrument that is called an ellipsometer and is based on the measurement of the change that takes place in ellipticity of reflected light produced by the deposited film. The apparatus is capable of measuring a film thickness within  $\pm 0.3$  Å, a sensitivity at least ten times greater than that obtained with the method based on light interference.

Rothen<sup>122</sup> in a series of papers has described experiments in which he deposited a film of protein on a metal slide and then covered this deposited layer of protein with a blanket of fatty-acid layers or of Formvar—a formal-

<sup>119</sup> W. D. Harkins, L. Fourt, and P. C. Fourt, *J. Biol. Chem.* 132, 111 (1940).

<sup>120</sup> K. B. Blodgett and I. Langmuir, *Phys. Rev.* 51, 964 (1937).

<sup>121</sup> A. Rothen, *Rev. Sci. Instruments* 16, 26 (1945); 20, 66 (1949).

<sup>122</sup> A. Rothen, *J. Biol. Chem.* 163, 345 (1946); 167, 299 (1947); 168, 75 (1947) *J. Am. Chem. Soc.* 70, 2732 (1948).



dehyde polyvinyl polymer); he was able to show that a specific protein deposited on top of the blanket was able to interact with the protein under the blanket. The specific protein was either an enzyme, such as trypsin or pepsin, in which case the underlying protein was digested or the specific protein was an antibody which reacted with the underlying antigen. The interaction was detected by the change in the thickness of the deposited film as measured with the ellipsometer. The thickness of the blankets could be increased to 200 Å, and yet apparent interaction through the blankets was observed. Rothen has interpreted his results in terms of long-range specific forces which were postulated to act through the blankets. Rothen's interpretation of his results have been criticized by Karush and Siegel,<sup>114</sup> by Singer,<sup>115</sup> and by Trurnit.<sup>116</sup>

The burden of this criticism is that the deposited blankets are irregular and provide holes through which the protein can diffuse; thus the specific protein can, in fact, come in direct contact with its substrate. The author has no confidence in the interpretations that Rothen has placed on his experiments.

#### FILMS AT OIL-WATER INTERFACES

Spread films have been studied at oil-water interfaces. Askew and Danielli<sup>117</sup> used a modified film-balance technique. On the other hand, Alexander, Teorell, and Aborg<sup>118</sup> spread the material at the interface in a fixed area and measured the change in film pressure by a surface-tension technique. The pressure was varied by increasing the amount of material at the interface. This last technique appears to be more convenient than the modified film balance. No very unexpected results were reported. The films tended to be more expanded than at the air-water surface.

Cumper and Alexander<sup>119</sup> have investigated spread monolayers of protein at an oil-water interface in respect to film pressure and film viscosity. They parallel these measurements with a study of these factors at an oil-water solution interface in which the protein was dissolved in the aqueous phase.

<sup>114</sup> F. Karush and B. M. Siegel, *Science* **108**, 107 (1948).

<sup>115</sup> S. J. Singer, *Federation Proc.* **8**, 251 (1949).

<sup>116</sup> H. J. Trurnit, *Science* **111**, 1 (1950).

<sup>117</sup> F. A. Askew and J. F. Danielli, *Trans. Faraday Soc.* **36**, 785 (1940).

<sup>118</sup> A. E. Alexander and T. Teorell, *Trans. Faraday Soc.* **35**, 727 (1939).

A. E. Alexander, T. Teorell, and C. G. Aborg, *Trans. Faraday Soc.* **35**, 1200 (1939).

<sup>119</sup> C. W. N. Cumper and A. E. Alexander, *Trans. Faraday Soc.* **46**, 235 (1950).

PROBLEMS AND QUESTIONS

1. What is a polar group; a non-polar group? Give examples of each.
2. Distinguish between work of adhesion, adhesion tension, and spreading coefficient.
3. What is the general and necessary condition for the validity of Langmuir's adsorption equation?
4. Water vapor uptake by 100 grams of dry protein was studied as a function of the relative water vapor pressure ( $P/P_0$ ) at 25° C. with the following results:

$P/P_0$	Grams Water	$P/P_0$	Grams Water
0.05	2.65	0.60	12.72
0.10	3.91	0.70	15.26
0.20	5.86	0.80	18.97
0.30	7.56	0.90	27.00
0.40	9.28	0.95	36.20
0.50	10.95		

Calculate the free energy of hydration of 100 grams of this protein, as well as the weight of the water adsorbed in the first layer. *Ans.:  $\Delta F = -956$  calories; 6.15 grams.*

5. 0.0134 mg. of a protein was spread on 5 per cent ammonium sulfate and the surface pressure in dynes per centimeter measured as a function of the area of the film in square centimeters with the following results at 25° C.

Film Pressure	Film Area	Film Pressure	Film Area
0.065	322	0.234	182
0.072	280	0.292	175
0.092	252	0.352	168
0.105	224	0.494	161
0.163	196		

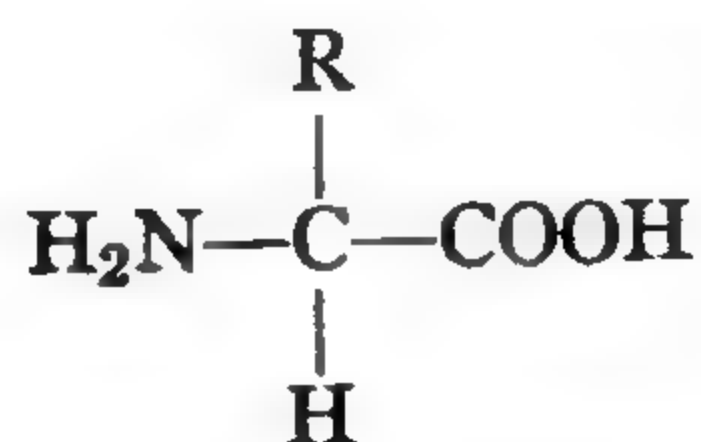
Calculate the molecular weight of this protein as well as its gaseous area in square meters per milligram. *Ans.: 36,000; 1.06 sq. m/mg.*

## BIOPOLYMERS

There occur in plants and in animals high-molecular-weight organic compounds which are polymers of smaller units. It has been customary to refer to such substances as colloids. The term was originated by Graham<sup>1</sup> to distinguish between the substances that diffuse rapidly which he called crystalloids and those substances that diffuse in solution much more slowly which he called colloids. The word was derived from the Greek *kolla*, meaning glue. Later, arbitrary size limits<sup>2</sup> were fixed for the particle and those substances whose particle diameter was greater than 1.0  $m\mu$  and less than 0.5  $\mu$  were considered to be colloids. The term colloid has never appealed to the author as a fortunate choice. After all, few biopolymers have properties that would justify their designation as glue. Another objection is the arbitrary size limits; these limits do not, in fact, bear any close relation to the change in properties of the system. If the particle shows marked asymmetry of shape, the situation becomes even more difficult and ambiguous; which of the dimensions of the particle are to be selected for classification? The name biopolymers appears to be more suited to our immediate needs. These substances are too diverse in character and too numerous to consider their molecular structure in detail and our concern will be along general lines.

## PROTEINS

The amino acids are the polymerizing units of proteins. There are some 20 naturally occurring amino acids with the general structure



where R is a group which determines the individuality of the amino acid.

<sup>1</sup> T. Graham, *Phil. Trans.* p. 183 (1861); *J. Chem. Soc. (London)* 17, 318 (1864).

<sup>2</sup> W. Ostwald, *An Introduction to Theoretical and Applied Colloid Chemistry*, John Wiley & Sons, New York, 1922.



This group may be a paraffin chain such as contained in valine and in the leucines. It may contain a carboxyl group such as does aspartic and glutamic acids or the R-group may have in it an amino group as does lysine or it may contain a non-ionogenic aliphatic hydroxyl as does threonine; there are other possibilities. The stereochemical configuration around the  $\alpha$ -carbon atom of all the amino acids derived from proteins has the same arrangement as the groups about the  $\alpha$ -carbon of L-lactic acid. The amino acids minus the elements of water from the  $\alpha$ -amino and  $\alpha$ -carboxyl groups are known as amino acid residues. In proteins these residues are connected by peptide bonds to produce peptide chains which may contain hundreds of amino acid residues. One of the most important problems in protein chemistry is the determination of the sequence of the amino acid residues in the peptide chains of proteins.<sup>1</sup> Owing to the massive R-groups as well as to the presence of polar groups—groups capable of hydrogen-bond formation<sup>2</sup>, peptide chains are stiff with a measure of rigidity. There is evidence from X-ray diffraction studies that many proteins have their peptide chains folded in a highly ordered arrangement in the molecule. Because of the specific folding of the peptide chain and of the enormous variety of sequence of amino acid residues in the peptide chain, proteins exhibit a far greater range of specific structures than do any other biopolymers. Diagrammatic sketches of stretched peptide chains occur in Chapter 7 and in Chapter 16.

### POLYSACCHARIDES

The three principal polysaccharides are starch, glycogen, and cellulose. The structural unit is the D-glucopyranose ring.

Starch exists in two structures, the linear form of which Schoch<sup>4</sup> calls the A-fraction which consists of straight chains of D-glucopyranose rings connected through the  $\alpha$ -1, 4 linkage and the B-fraction which contains 50 to 70 branches each 25 to 30 glucose units in length. These branches are connected through the  $\alpha$ -1, 6-linkage. Glycogen structure resembles that of B-starch and consists entirely of branched molecules which vary considerably in size, depending largely on the source of the glycogen. The glucose residues are joined by  $\alpha$ -1, 4-linkages, and branching occurs through  $\alpha$ -1, 6-linkages. Cellulose is the principal structural element of plants and is composed of D-glucopyranose residues joined by  $\beta$ -1, 4-glycosidic linkages with very little branching. Shown in Fig. 1 are D-glucopyranose units connected as they are in starch A-form.

Glycogen, starch, and cellulose are richly endowed with hydroxyl groups which can bind water through hydrogen bonds.

Numerous polysaccharide-like compounds exist other than the three

<sup>1</sup> F. Sanger, *Cold Spring Harbor Symposia Quant. Biol.* 14, 153 (1949).

<sup>4</sup> T. J. Schoch, *Advances in Carbohydrate Chem.* 1, 247 (1945).

mentioned above. The plant gums form a large class embracing gum arabic, gum tragacanth, and others. Agar and Irish moss mucilage are also complex polysaccharides and occur as sulfuric acid esters. Chitin, a nitrogen containing polysaccharide, occurs in certain fungi and is common among invertebrates. Meyer and Mark<sup>5</sup> conclude from X-ray diffraction studies on chitin that units of acetyl glucosamine are united by  $\beta$ -1, 4-linkages to form long chains. An important biopolymer is hyaluronic acid which contains an equal number of N-acetyl glucosamine and glucuronic

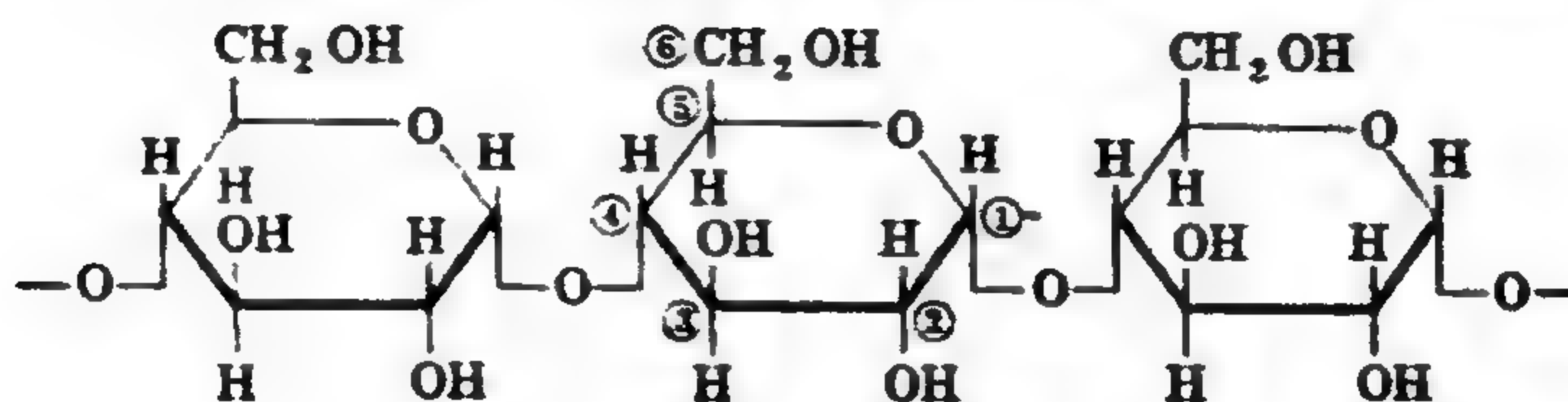


FIG. 1. D-glucopyranose units connected as in starch.

acid residues.<sup>6</sup> The molecular weight of hyaluronic acid is approximately 200,000 to 400,000. The substance serves as a cementing material for body cells and forms viscous barriers preventing the spread of infectious agents.

### NUCLEIC ACIDS

Nucleic acids are polymers of nucleotides. A nucleotide contains a pentose sugar (ribose or deoxyribose), phosphoric acid and a purine or pyrimidine base. Adenosine monophosphate (adenylic acid) is a nucleotide. The mononucleotides are linked to each other in nucleic acid through the pentose and phosphoric acid residues, and the purine and pyrimidine residues are not included in the backbone of the chain. The nucleic acid chain has the structure



where P is the phosphoric acid, S is the sugar, and B is the purine or pyrimidine base. By means of differential staining methods, it has been concluded<sup>7</sup> that the nuclei of cells contain chiefly deoxyribonucleic acid, whereas the cytoplasm contains chiefly ribonucleic acid. Owing to the high phosphoric acid content of nucleic acid, they are very acidic and show a marked tendency to combine with basic proteins. Nucleic acid molecules are very elongated and thread-like, giving rise to highly viscous solutions. The molecular weight investigation on the sodium salt of thymonucleic

<sup>5</sup> K. H. Meyer and H. Mark, *Ber.* 61, 1936 (1928).

<sup>6</sup> K. Meyer, E. Smith, and M. Dawson, *J. Biol. Chem.* 128, 319 (1939).

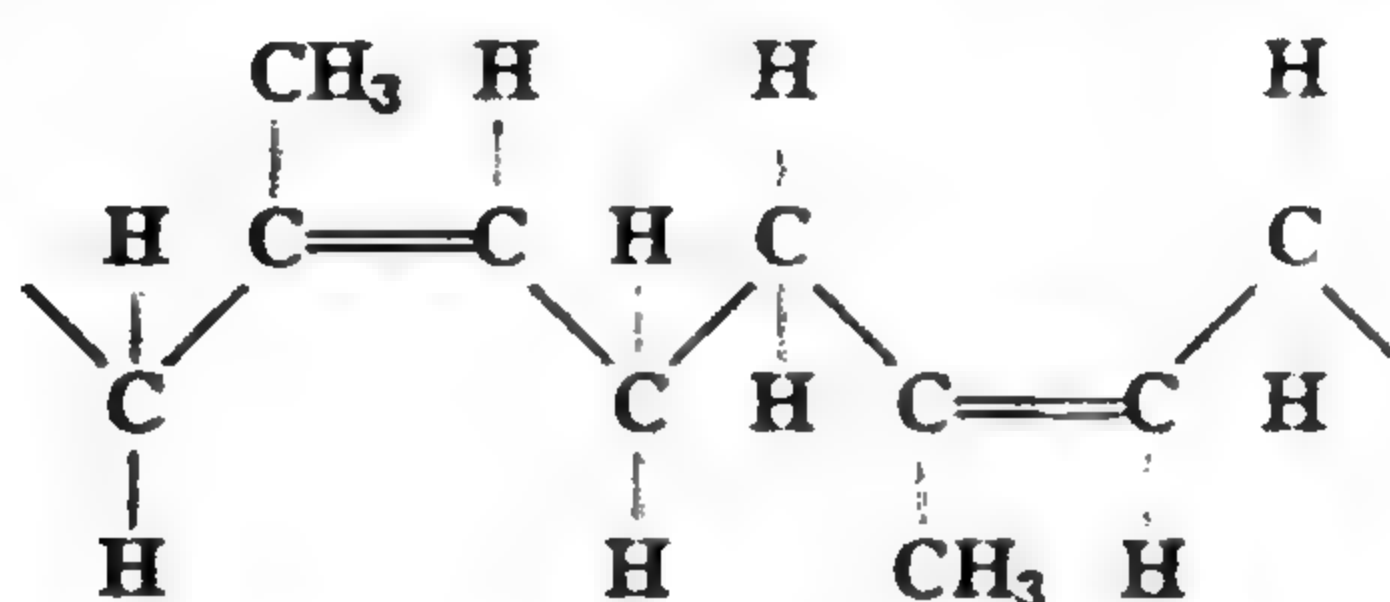
<sup>7</sup> I. Davidson, C. Waymouth, *Biochem. J.* 38, 379 (1944); *J. Physiol.* 105, 191 (1946).



acid in aqueous solution by the light-scatter method<sup>8</sup> showed the thread-like molecules to be coiled in a more or less random fashion with a molecular weight greater than  $24 \times 10^6$ .

### RUBBER

Rubber is a polymer of isoprene and occurs in the latex of the Euphorbiaceae, Apocynaceae, and Moraceae families. Particularly good rubber is given by trees of the species *Hevea*. Rubber is distinguished by the complete absence of polar groups and of massive side-chain residues with the result that the molecular chains are very flexible leading to the well-known elastic properties of rubber. The isoprene units are connected as follows:



In rubber, the groups about the double bond are *cis* to each other, whereas in gutta-percha, another polymer of isoprene obtained from the latex of tropical trees, the groups are *trans* to each other. The hydrocarbon chains in the *trans* configuration fit together in parallel, whereas the *cis* form in rubber does not fit snugly in the solid structure, leading to kinking of the chains and to long-range elasticity.

### SOLUTIONS OF BIOPOLYMERS

Many biopolymers can be put into aqueous solution. The methods for the determination of the shapes and sizes of such dissolved molecules will engage our attention for the next few chapters. Some biopolymers' molecules are branched, whereas others are linear or can be stretched into a linear form. The natural tendency of a linear molecule is to fold and to approach what is known as a random coil.<sup>9</sup> This is the most probable configuration of the molecule; i.e., the entropy is greatest in this form. The folding arises from the random kinking of the links in the chain about valence bonds and results from the heat motion (Brownian motion) of the molecular chain. A random coil has the form of an ellipsoid whose axes on the average bear the approximate ratios of 6:2.3:1 to each other. The distance between the ends of the molecular chain is proportional to the square root of the number of flexible links in the chain. The tightness of such a coil depends on the solvent; the more suitable the solvent, the more

<sup>8</sup> D. B. Smith and H. Shaffer, *Can. J. Research* 28, 96 (1950).

<sup>9</sup> W. Kuhn, *Kolloid-Z.* 68, 2 (1934).



nearly does the coil approach a random condition. A poor solvent leads to shrinkage of the coil with the displacement of solvent between the coiled polymer chains.

The presence of interacting groups in the polymer causes the form of the fold to depart from that of a random coil. As has been noted, many protein molecules show very specific folding of the peptide chains with a pronounced degree of order. Such folding arises, no doubt, in part from the way in which the proteins are synthesized in the living cell, and also in part from the interaction of the polar amino acid residues with each other. Between the random coil and the specific fold of proteins is every degree of order and disorder.

### STABILITY OF BIOPOLYMER SOLUTIONS

Owing to heat motion of the particles there occurs, even in moderately dilute solutions of biopolymers, an enormous number of collisions in a short interval of time. If each collision resulted in cohesion between the particles, the sol would precipitate very rapidly. Smoluchowski<sup>16</sup> has calculated the rate of disappearance of particles if all collisions were effective, and he derived the following equation

$$N = \frac{N_0}{1 + t/t_{1/2}} \quad (1)$$

where  $N$  is the number of particles per cubic centimeter at any time  $t$ , and  $N_0$  is the original number of particles per cubic centimeter.  $t_{1/2}$ , the time required to reduce the number of particles by one-half, is

$$t_{1/2} = \frac{3\eta}{N_0 4kT} \quad (2)$$

where  $\eta$  is the coefficient of viscosity of the dispersions medium,  $k$  is Boltzmann's constant ( $1.38 \times 10^{-16}$  erg per degree), and  $T$  is the absolute temperature. Thus, according to Smoluchowski, the rate of precipitation, provided that every collision is effective, is independent of size and specific character of the particles. Smoluchowski's equation has been found to be valid for many suspensions. However, if the suspension is pronouncedly polydispersed, it coagulates at a faster rate than is predicted by this equation and rod-shaped particles show a higher rate of precipitation than do spherical particles.

In spite of the high collision rate of biopolymers in solutions, such solutions are, in general, reasonably stable. They owe this stability to two factors: (1) hydration of the polar groups and (2) the electrostatic charges on the surface of the particles. It is easy to see in a qualitative fashion why

<sup>16</sup> M. v. Smoluchowski, *Physik. Z.* 17, 557, 583 (1916).

hydration should increase the stability of such a solution. The polar groups which would be most potent in bringing about cohesion between the particles are blocked by water molecules. The water held on polar groups of one particle has but little more attraction for water bound to another particle than for free water in the system. Accordingly, little energy is gained by the coalescence of two particles, and the system is stabilized.

It has long been recognized<sup>11</sup> that the electrostatic charge on the particles provides stability; to bring two charged particles together requires the expenditure of energy to overcome the electrostatic repulsion, and the role of the electrostatic charges in maintaining the stability of a suspension of colloided gold particles in which hydration of the particles does not occur is particularly notable. Such sols can be precipitated by quite small amounts of electrolytes, and the valence of the ion of opposite sign to the colloid is very important; divalent ions are much more effective than are monovalent ions, and the trivalent ions are more effective than divalent. This is a qualitative expression of the Hardy-Schulze rule.

It was considered that the  $\zeta$ -potential of the particles is a measure of the stability conferred by the electrostatic charges. This, however, proves to be a highly complex affair and has been the subject of considerable experimental and theoretical work. This topic has been discussed in Chapter 9 and will be dealt with again in Chapter 12.

## SOLUBILITY

The solution of any substance is the reflection of three energy changes.

1. The molecules must be removed from the surrounding molecules in the solid phase and the forces of cohesion overcome.
2. A hole must be made in the solvent to receive the dissolved molecule. This requires the expenditure of energy and is related to the energy of vaporization of the solvent.
3. The dissolved molecule is then inserted in the hole in the solvent. Energy is gained from this process, and the amount of energy is related to interaction between the solvent and the solute.

There is a natural tendency for a solid material to dissolve in a liquid just as two gases, when brought in contact, tend to mix; the entropy of the system is increased by the mixing process. For the entropy of mixing of  $n'$  solvent with  $n''$  polymeric solute molecules

$$\Delta S = -k[n' \ln r_1 + n'' \ln r_2] \quad 3.$$

where  $k$  is Boltzmann's constant and  $r_1$  and  $r_2$  are the volume fractions of the components, solvent, and polymeric solute, respectively.

<sup>11</sup> W. B. Hardy, *Proc. Roy. Soc. (London)* 66, 110 (1900).



Onsager,<sup>12</sup> however, has concluded that there are situations where the entropy of a system is increased by a separation of a solid phase from a solution. Consider, for example, a solution of elongated rod-like molecules, such as those of tobacco mosaic virus protein. If the solution is so concentrated that the individual molecules cannot execute random rotation freely, then there will be a gain in entropy of the system if some of the solute molecules precipitate in an ordered arrangement. There is a dilution of the solution thereby, and the molecules in solution can then acquire full random rotary movement. The formation of two phases by tobacco mosaic virus has been studied by Oster,<sup>13</sup> using light-scatter methods.

Possibly the effect noted by Onsager is responsible for tactoid formation. Tactoids are large boat- or spindle-shaped particles which have been observed to form in concentrated solutions of iron oxide and of vanadium pentoxide, and also in solutions of several dyes. Such aggregations of particles were called tactoids by Zocher,<sup>14</sup> who first described them. Tactoids are optically anisotropic as revealed by the polarizing microscope. This indicates that they are composed of oriented particles and the direction of orientation coincides with the long axis of the tactoid.

Bernal and Fankuchen<sup>15</sup> report that tobacco mosaic virus protein exhibits well-defined tactoids. The larger the virus tactoids, the more nearly they approach a spherical form; the smaller they are, the more cylindrical they become.

Many factors influence the solubility of biopolymers; among these may be mentioned: temperature, electrolytes, nature of polymer, and nature of solvent. The solubility of proteins has been studied in far greater detail than has that of the other biopolymers, and, accordingly, it seems appropriate to draw our examples from this class of substance.

#### TEMPERATURE

The process of solution can be regarded in a sense as a melting of the solute, and the variation of the solubility with temperature should be closely related to the heat of solution; indeed it can be shown that

$$\frac{d \ln S}{dT} = \frac{\Delta H}{RT^2} \quad (4)$$

where  $\Delta H$  is the heat absorbed when one mole of the solute goes into solution and  $S$  is the solubility of the solute in the solvent. If heat is evolved on solution, then the solubility will decrease as the temperature is increased.

<sup>12</sup> L. Onsager, *Ann. N. Y. Acad. Sci.* 51, 627 (1949).

<sup>13</sup> G. Oster, *J. Gen. Physiol.* 33, 445 (1950).

<sup>14</sup> H. Zocher, *Z. anorg. Chem.* 147, 91 (1925).

<sup>15</sup> I. D. Bernal and I. Fankuchen, *J. Gen. Physiol.* 25, 111 (1941).



The heat of solution ( $\Delta H$ ) is not always constant with the change in temperature and can indeed change sign over a sufficient temperature range. For example, Sørensen<sup>16</sup> in his classic work on the solubility of egg albumin in the presence of 2.14 *M* ammonium sulfate found the following solubilities for this protein in grams per liter at the indicated temperatures: 0° C., 3.18 grams; 12° C., 2.09 grams; 20° C., 1.81 grams; 29° C., 2.24 grams.

### MOLECULAR WEIGHT

As we have seen under the discussion of Traube's rule (Chapter 10), the energy required to transfer a mole of a member of a homologous series (fatty acids) from a solution to the surface is directly related to the molecular weight of the member. Many biopolymers exist as mixtures differing principally in molecular weight. It should, therefore, be possible to fractionate these polymeric species from each other on the basis of solubility as a result of these differences in molecular weight. Flory<sup>17</sup> has investigated this matter and concludes that the efficiency of separation depends on the ratio of the volumes of the supernatant and the precipitated phases. In order to attain a high value for this ratio, very dilute solutions must be employed and the higher the molecular weight the greater is the dilution required for the same sharpness of separation.

### INFLUENCE OF SALTS

As was pointed out in the second chapter, the molar free energy of a solute in equilibrium with some of its solid phase is equal to that of the solid. It follows that the activity of a solute in a saturated solution at a given temperature is a constant. Accordingly, by measuring the solubility as a function of the salt content of the solution, the variation of the activity coefficient of the solute can be determined. This method of study has been widely applied by physical chemists to numerous substances whose solubility is limited. Exactly the same type of study can be done on proteins and other high-molecular-weight substances, and the variation of the activity coefficient of such substances in solution can be measured as a function of the electrolyte content of the solution. Mellanby,<sup>18</sup> as a result of such studies on the serum globulin, was able to anticipate the principle of ionic strength formulated in 1921 by Lewis and Randall. Mellanby found that dilute solutions of ions with equal valences, whether positive or negative, were equally effective in increasing the solubility of the globulin, and the efficiencies of ions of different valences were directly proportional to the square of their valences.

<sup>16</sup> S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg* 12, 213 (1915-17).

<sup>17</sup> P. J. Flory, *J. Chem. Phys.* 12, 425 (1944).

<sup>18</sup> J. Mellanby, *J. Physiol.* 33, 338 (1905).

The influence of salts on the solubility of proteins is rather complicated. In general, dilute salt solutions increase the solubility of proteins. Indeed, it will be recalled that euglobulins are insoluble in pure water but become soluble in dilute salt solutions. As the salt concentration is increased beyond a certain point, however, protein solubility decreases. In this region a lyotropic series of the ions is usually rather conspicuous. In Fig. 2 is

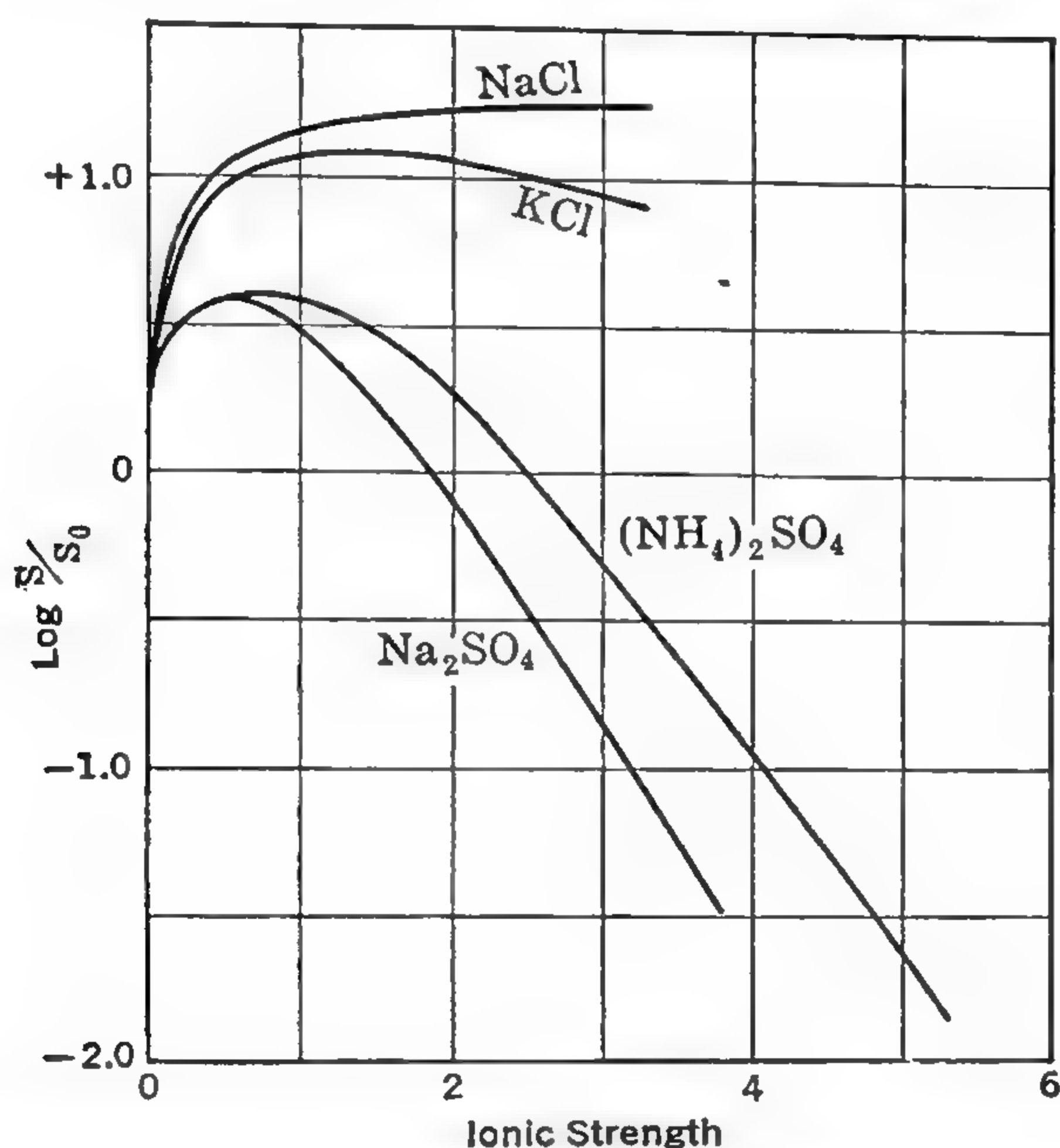


FIG. 2. Solubility of carboxyhemoglobin in aqueous salt solutions.  $S_0$  is the solubility in pure water and  $S$  is the solubility in the salt solution. (Data of A. A. Green.)

shown<sup>19</sup> the variation of the solubility of carboxyhemoglobin as a function of the ionic strength at 25° C.

Scatchard and Kirkwood<sup>20</sup> and later Kirkwood<sup>21</sup> extended the Debye-Hückel theory of interionic attraction to dipolar ions. They concluded that the activity coefficient of a zwitter ion should decrease in the presence of a neutral salt and that the negative logarithm of the activity coefficient of the zwitter ion should be directly proportional to the ionic strength of the solution. Their conclusions apply only to dilute salt solutions.

<sup>19</sup> E. J. Cohn, *Chem. Revs.* **19**, 241 (1936).

<sup>20</sup> G. Scatchard and J. G. Kirkwood, *Physik. Z.* **33**, 297 (1932).

<sup>21</sup> J. G. Kirkwood, *J. Chem. Phys.* **2**, 351 (1934).

INFLUENCE OF  $pH$ 

The solubility of proteins is a marked function of the  $pH$ ; the solubility being least in the isoelectric zone and increasing on both the acid and the basic side. Figure 3 shows the variation of the solubility of  $\beta$ -lactoglobulin as a function of  $pH$ .<sup>22</sup>

It is easy to offer a qualitative explanation of the influence of  $pH$  on the solubility of a protein. The solubility of any substance is the resultant of

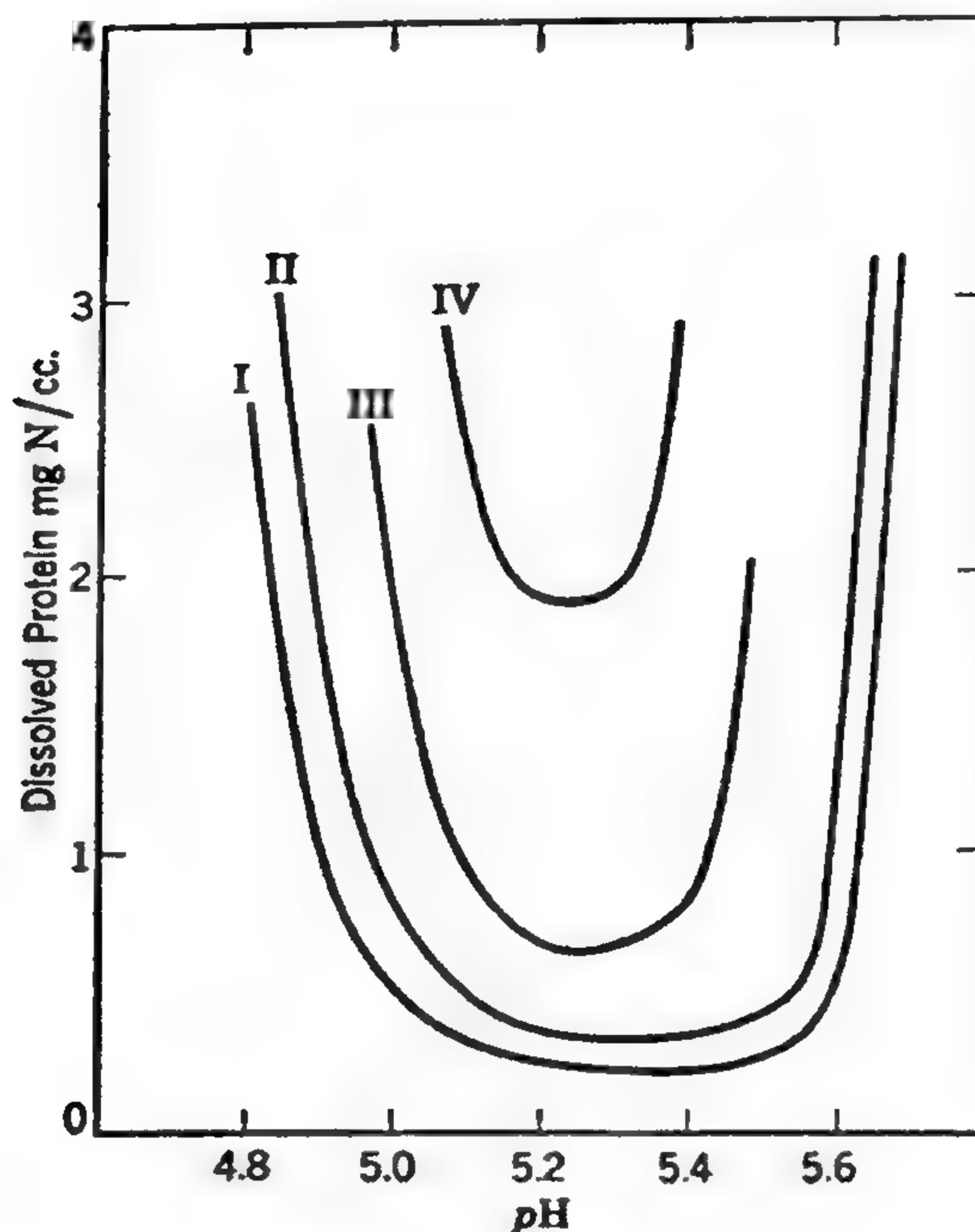


FIG. 3. Solubility of  $\beta$ -lactoglobulin as a function of  $pH$ . Curve I 0.001 ionic strength, curve II 0.005 ionic strength, curve III 0.01 ionic strength, curve IV 0.02 ionic strength (Grönwall.)

the balance of the attraction of the solute molecules for each other, which tends to prevent solution, and the attraction of the solvent molecules for the solute, which tends to promote solution. At the isoelectric point the attraction of the protein molecules for one another is maximal. If the  $pH$  is shifted away from the isoelectric point, the protein acquires a net charge. This decreases the attraction of the protein molecules for each other, and consequently the solubility of the protein tends to increase.

Linderström-Lang<sup>23</sup> has proposed a theory of the solubility of proteins

<sup>22</sup> A. Grönwall, *Comp. rend. trav. lab. Carlsberg* 24, 185 (1942).

<sup>23</sup> K. Linderström-Lang, *Arch. Biochem.* 11, 191 (1946).



as a function of the  $pH$  which enables him to calculate the molecular weight of the protein from the variation of the solubility with  $pH$  and with the number of protons bound to the protein. He arrives at the conclusion that, for a given number of protons bound, the logarithm of the solubility is proportional to the molecular weight of the protein.

### NATURE OF SOLVENT

The solvent action of organic molecules upon amino acids and peptides increases with increase in the number of non-polar paraffin side chains in the zwitter ion.

The charged groups of proteins give rise to electrostatic forces between molecules. Addition of organic solvents, as does increase in temperature, leads to a decrease in the dielectric constant of the solution, thus increasing the magnitude of all electrostatic forces.

Many proteins show alcohol solubility. For example, egg albumin is soluble in 18 per cent ethyl alcohol at room temperature; at lower temperatures the per cent of alcohol can be greatly increased without any damage to the protein.

As is well known, alcohol fractionation at low temperatures permitted the separation of blood serum protein on a large scale by Cohn and his group.<sup>24</sup>

### SOLUBILITY TEST FOR PURITY

Sørensen<sup>16</sup> showed that egg albumin at fixed temperature, pressure, salt concentration, and  $pH$ , behaved very nearly like a single component, showing a solubility very nearly independent of the amount of the solid phase in concentrated ammonium sulfate solutions.

The work of Northrop and his collaborators<sup>25</sup> has shown that a number of proteins can be obtained in sufficient purity to fulfill rigorously the requirements for a pure single component.

In order to measure the solubility of a protein, varying amounts of solid protein are placed in a series of test tubes. The test tubes are completely filled with solvent and the tubes stoppered. No air is allowed in the tubes because of danger from surface denaturation of the protein. The tubes are rotated slowly and are stirred by marbles which are placed in them along with the protein. The time of stirring in a constant-temperature bath necessary to attain equilibrium must be determined by experiment. The solutions are then filtered, and the amount of protein in the filtrate is determined. The amount of protein in solution is then plotted against the

<sup>24</sup> E. J. Cohn et al., *J. Am. Chem. Soc.* 68, 459 (1946).

<sup>25</sup> J. H. Northrop, M. Kunitz, and R. M. Herriott, *Crystalline Enzymes*, Columbia University Press, New York, 1948.

total amount of protein present. Figure 4 shows diagrammatically the results that may be obtained.

It is well known that, for a pure chemical compound, the solubility is independent of the amount of solid phase present. This is shown in Fig. 4.1. No solid phase is present until the break in the line is reached at which point no more of the added solid dissolves.

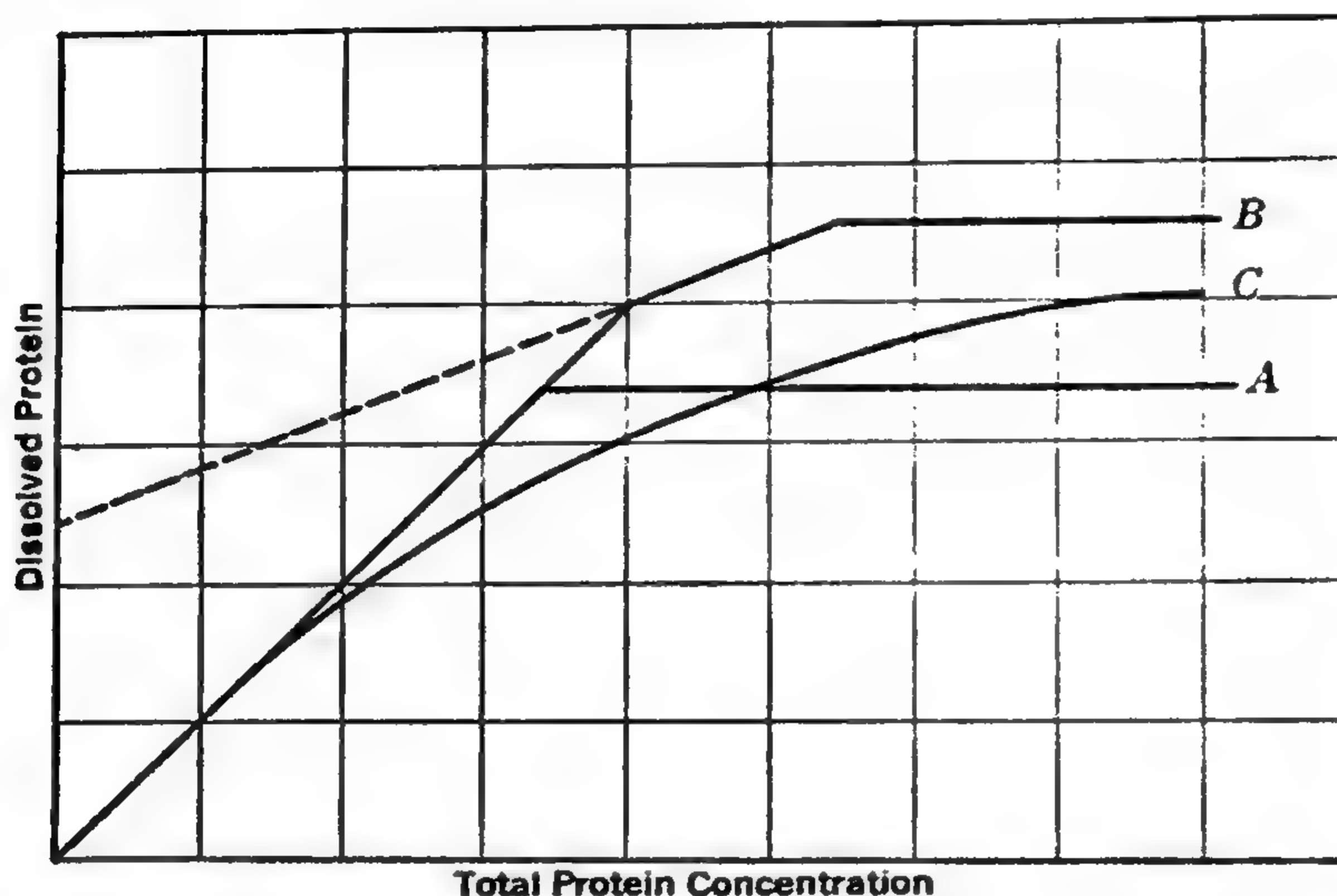


FIG. 4. Diagrammatic solubility curves. (A) Solubility curve of a single pure protein. (B) Solubility curve of a mixture of two proteins. (C) Solubility curve of solid solution of two or more protein components.

### INTERACTION OF BIOPOLYMERS

In a number of instances interaction between biopolymer molecules occurs. For example, nucleic acid reacts with proteins in the appropriate pH zone where the protein carries a positive charge. Somewhat the same kind of reaction is that occurring between the strongly basic protamine and insulin, a combination that has found wide clinical favor. In both these examples the interaction is primarily electrostatic and involves the attractive forces between oppositely charged particles. This type of combination is by no means the only one possible. Enzymes must combine with the substrate before they can act. Frequently, the enzyme and substrate molecules carry the same sign of charge.

A reaction between biopolymers that has been extensively investigated is that between antibody and antigen in a precipitation reaction. It is well known that, if a protein foreign to an animal be injected into the animal, the animal's serum acquires the power to precipitate the foreign protein. The injected protein is called an antigen, and the protein in the serum that



can precipitate the antigen is called an antibody. This is an example of two hydrophilic molecules both of which are negatively charged precipitating each other. The antibody exactly "fits" the antigen molecule; polar groups of the antibody must be able to come in contact for the antigen and antibody to unite to form the precipitate. According to Breinl and Haurowitz,<sup>26</sup> the structure of the antibody is complementary to that of the antigen.

Characteristically, there is a zone of maximum precipitation in which the ratio of antibody to antigen is most favorable for precipitation and, if the ratio be increased or decreased, the precipitate tends to dissolve. Kendall<sup>27</sup> and Heidelberger<sup>28</sup> were able to derive a quantitative expression for the amount of precipitate formed in terms of the amounts of antibody and antigen added. Pauling, Campbell, and Pressman<sup>29</sup> were able to present a somewhat more elegant derivation of this same expression.

It has long been known that, in antigen-antibody precipitates, molecules of antibody in the zone of maximum precipitation are present in larger numbers than those of the antigen. This raises the question as to the valence of the antibody and antigen molecules. Some workers feel<sup>30</sup> that antibodies are univalent, whereas others are of the opinion that antibodies are at least bivalent.<sup>31</sup> There is general agreement that antigens are polyvalent and their valence is principally determined by the number of antibody molecules that can be packed around the antigen; the larger the antigen, the greater is its valence.

### THE GOLD NUMBER

The so-called protective action of hydrophilic colloids on the stability of hydrophobic suspensions has received considerable study and is, among other things, the basis for a medical test of diagnostic value. It has been known for a long time that hydrophobic suspensions, when treated with various hydrophilic suspensions, become much more stable towards electrolytes. In 1857 Michael Faraday demonstrated his gold sol to the Royal Society. The sol was placed in the museum of the Society and remains there to the present day. Such longevity in a gold sol is astonishing. Investigation has shown that Faraday had added a protective colloid to his gold sol.

Colloidal silver and colloidal silver oxide have pronounced bactericidal properties, and the "Argyrol" of medicine is a colloidal silver protected by protein split-products.

\* F. Breinl and F. Haurowitz, *Z. physiol. Chem.* 192, 45 (1930).

\* F. E. Kendall, *Ann. N. Y. Acad. Sci.* 43, 85 (1942).

\* M. Heidelberger, *Chem. Revs.* 24, 323 (1939).

\* L. Pauling, D. H. Campbell, and D. Pressman, *Physiol. Revs.* 23, 203 (1943).

\* F. Haurowitz, *Progress in Biochemistry*, Interscience Publishers, Inc., New York, 1950.

\* L. Pauling, *J. Am. Chem. Soc.* 62, 2643 (1940).



The protective action of a hydrophilic substance is usually expressed in terms of the gold number. The gold number is defined as that weight of hydrophilic colloid in milligrams that is just insufficient to prevent a change in color from red to violet when 1 cc. of a 10 per cent sodium chloride solution is added to 10 cc. of a red gold sol to which the hydrophilic substance has been added. Gold numbers found by Gortner<sup>22</sup> for several hydrophilic substances are shown in Table 1.

TABLE 1  
GOLD NUMBERS OF SOME HYDROPHILIC SUBSTANCES

Substance	Gold Number
Gelatin	0.005–0.0125
Egg albumin*	0.08–0.10
Protalbinic acid	0.15–0.20
Lysalbinic acid	0.10–0.125
Gum arabic	0.10–0.125
Dextrin (British gum)	125–150
Soluble starch	10–15

\* Highly purified egg albumin has a gold number of about 7. The presence of globulins greatly lowers the gold number.

The gold number of the cerebrospinal fluid was introduced into medicine a number of years ago as a diagnostic aid. Actually more valuable information is obtained by plotting out a curve for the spinal fluid. A series of ten test tubes is prepared, in the first of which is placed 1 cc. of the spinal fluid which has been diluted 1 to 10 with 0.4 per cent NaCl solution. In the next tube is placed 1 cc. of a 1 to 20 dilution, in the third 1 cc. of a 1 to 40 dilution, and so on. To each tube is added 5 cc. of a red gold sol. and the mixtures are shaken. The tubes are observed after 24 hours. The several colors are plotted as ordinate against the concentration of the spinal fluid as abscissa. A curve is obtained which is characteristic for certain types of diseases. The colors are numbered as follows: cherry red, 0; bluish red, 1; reddish blue, 2; deep blue, 3; pale blue, 4; colorless, indicating complete coagulation, 5. Figure 5 shows several such curves.

If the concentration of the hydrophilic substance is quite low, it is sometimes found that hydrophilic material not only does not protect the hydrophobic colloid but may even sensitize it to electrolytes. Sometimes this sensitizing action can be explained as a flocculation of oppositely charged colloids; however, it has happened that a hydrophobic suspensoid is sensitized by a hydrophilic colloid bearing the same sign of charge. The explanation of such action seems to lie along lines first suggested by Zsigmondy.

<sup>22</sup> R. A. Gortner, *J. Am. Chem. Soc.* 42, 595 (1920).

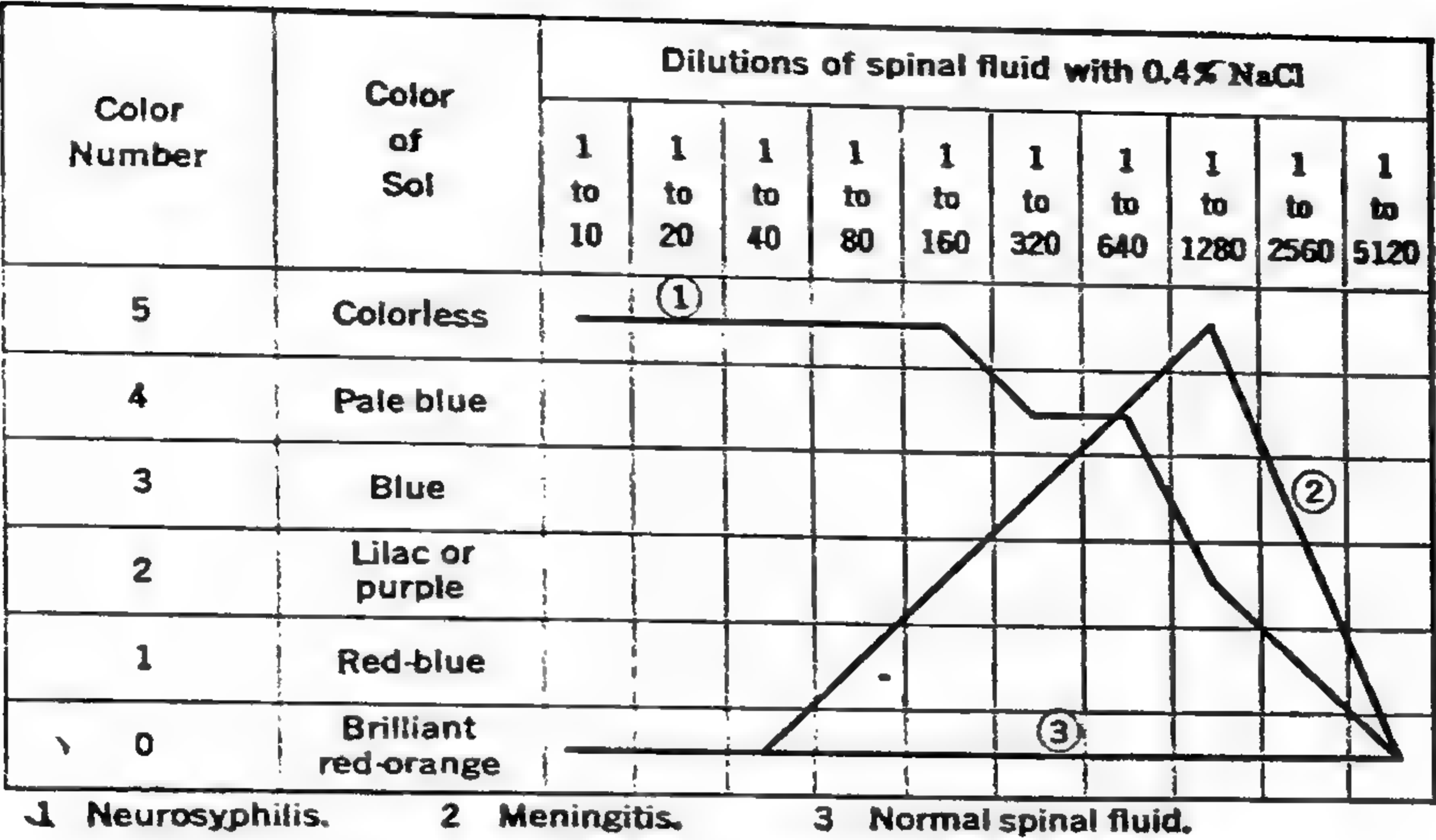


FIG. 5. Characteristic gold sol curves for cerebrospinal fluid.

The two colloids exhibit an appreciable tendency to unite irrespective of their charge. At low concentrations of the hydrophilic colloid, the hydrophobic colloid will be enveloped by the hydrophobic colloid, whereas, in



FIG. 6. Diagrammatic representation of the conditions for sensitization and protection of a hydrophobic sol. The circles represent hydrophilic particles; the squares, hydrophobic particles.

In the presence of an excess of hydrophilic colloid, the hydrophilic colloid envelops the hydrophobic colloid. The first situation leads to sensitization; the second, to protection. This relation is diagrammed in Fig. 6.

QUESTIONS

1. Characterize a random coil.
2. What factors govern the rate of collision of particles in solution?
3. Explain in general terms why a moderate salt concentration tends to increase the solubility of a biopolymer, whereas a higher one tends to decrease solubility.
4. Explain how it is possible to test the purity of a biopolymer by solubility measurements.

## OSMOTIC PRESSURE and RELATED PROPERTIES

Some properties of solutions depend primarily on the number of solute molecules present. Such properties are known as colligative properties. They are to be contrasted with properties that depend on the nature of the solute molecules and are called constitutive properties. Some colligative properties of solutions are the vapor-pressure lowering of the solvent, the freezing-point depression of the solvent, and the osmotic pressure.

### VAPOR-PRESSURE LOWERING

As is well known, water at any given temperature and pressure exerts a definite vapor pressure. If the temperature is raised sufficiently, a temperature will be found at which the aqueous vapor pressure equals the atmospheric pressure and the water boils. If some solute is added to the water, the vapor pressure of water is found to be less than that of pure water at the same temperature. The boiling point of water is, accordingly, increased by the addition of the solute.

Why a solute reduces the vapor pressure of the solvent and also why this lowering is proportional to the mole fraction of the solute may be seen from the following argument: Consider unit surface area of the pure solvent. The rate of exit of solvent molecules is equal, at constant temperature, to some constant  $a$ , while the rate of condensation of the vapor into the surface of the solvent is  $bP_0$ , where  $b$  is a constant and  $P_0$  is the vapor pressure of the pure solvent. At equilibrium, these two rates must be equal and

$$a = bP_0 \tag{1}$$

If we add  $n_2$  moles of the solute to  $n_1$  moles of the solvent, the rate of evaporation of solvent molecules from unit area of surface of solution is proportional to the mole fraction of the solvent and is, accordingly,  $n_1/(n_1 + n_2)$ . The rate of condensation into unit area of the surface is



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$P$ , where  $P$  is the vapor pressure of the solvent. At equilibrium these two rates are equal and

$$\frac{dn_1}{n_1 + n_2} = bP \quad (2)$$

Eliminating  $a$  and  $b$  between equations 1 and 2 and rearranging, we have

$$\frac{P}{P_0} = \frac{n_1}{n_1 + n_2} = N_1 \quad (3)$$

or

$$P = P_0 (1 - N_2) \quad (4)$$

and

$$\frac{P_0 - P}{P_0} = N_2 \quad (5)$$

Equations 3, 4, and 5 are expressions of Raoult's law.

Suppose that we make up a molal solution of some substance in water. The mole fraction of the solute in such a solution is, as we have seen in Chapter 2, equal to 0.0177. The vapor pressure of pure water at 25° C. is 23.75 mm. of mercury. Substituting these data in equation 5, we find

$$\begin{aligned} P_0 - P &= 23.75 \times 0.0177 \\ &= 0.420 \text{ mm. Hg} \end{aligned} \quad (6)$$

The molecular weight of a compound may be determined by measuring the vapor-pressure lowering of the solvent produced by the addition of a solute. If  $x_2$  grams of solute of molecular weight  $M_2$  are dissolved in  $x_1$  grams of solvent whose molecular weight is  $M_1$ , then the respective number of moles of each are

$$n_2 = \frac{x_2}{M_2} \quad \text{and} \quad n_1 = \frac{x_1}{M_1} \quad (7)$$

The Raoult equation becomes

$$\frac{P_0 - P}{P_0} = \frac{x_2 M_1}{x_1 M_1 + x_2 M_2} \quad (8)$$

In dilute solutions where  $n_1 \gg n_2$ , equation 8 reduces to

$$\frac{P_0 - P}{P_0} = \frac{x_2 M_1}{M_2 x_1} \quad (9)$$

Suppose that we dissolve 1 gram of a substance whose molecular weight is 10000 in 100 grams of pure water. what would be the vapor-pressure lowering?

At 25° C. we have

$$P_0 - P = \frac{23.75 \times 18}{10,000 \times 100} \quad (10)$$

$$= 0.000427 \text{ mm. Hg} \quad (10a)$$

The vapor-pressure lowering produced by the material with a molecular weight of 10,000 is much too small to be of use as a method for the determination of its molecular weight. The mere trace of a low-molecular-weight impurity would invalidate such measurements. Vapor-pressure lowering is quite unsuited for the determination of the molecular weights of proteins.

Barger<sup>1</sup> described what he called an isopiestic method for the estimation of molecular weights by vapor-pressure lowering. This method compares the tendency to distil from a solution of higher vapor pressure to one having a lower vapor pressure in a closed space. The change in size of drops of a standard solution and of the solution of unknown vapor pressure is compared. The drops are placed in a sealed capillary with air space between them.

The Hill method is another way in which the vapor pressure of a solution may be measured.<sup>2,3,4</sup> The apparatus consists of a sensitive thermocouple, the thermojunctions of which are made in the form of a loop. Upon one loop is placed a drop of the sample, and upon the other loop is placed a drop of a reference salt solution. Condensation into or evaporation from the drop of unknown will cause the thermojunction upon which it rests to become warmer (or cooler); thus the vapor pressure of the sample can be compared with that of a series of standard reference salt solutions.

## DEPRESSION OF THE FREEZING POINT

When a solute is added to a liquid, the freezing point of the solution is found to be less than that of the solvent. The experimental technique of measuring freezing-point depressions has been developed and refined. An accurate and sensitive thermometer is used by which the freezing point of the solution can be read to a thousandth of a degree. To understand the relation between freezing-point lowering and the vapor-pressure lowering consider Fig. 1.

$T_0 - T$  is evidently the freezing-point lowering and  $P_0 - P$  is the vapor-pressure lowering of the solution produced by the addition of the solute. For dilute solutions the vapor-pressure curves are almost parallel, and under

<sup>1</sup> G. Barger, *J. Chem. Soc.* 85, 286 (1904).

<sup>2</sup> E. J. Baldes, *J. Sci. Instruments* 11, 223 (1934).

<sup>3</sup> R. R. Roepke and E. J. Baldes, *J. Biol. Chem.* 126, 349 (1938).

<sup>4</sup> M. N. Fineman and J. W. McBain, *J. Phys. & Colloid Chem.* 52, 881 (1948).

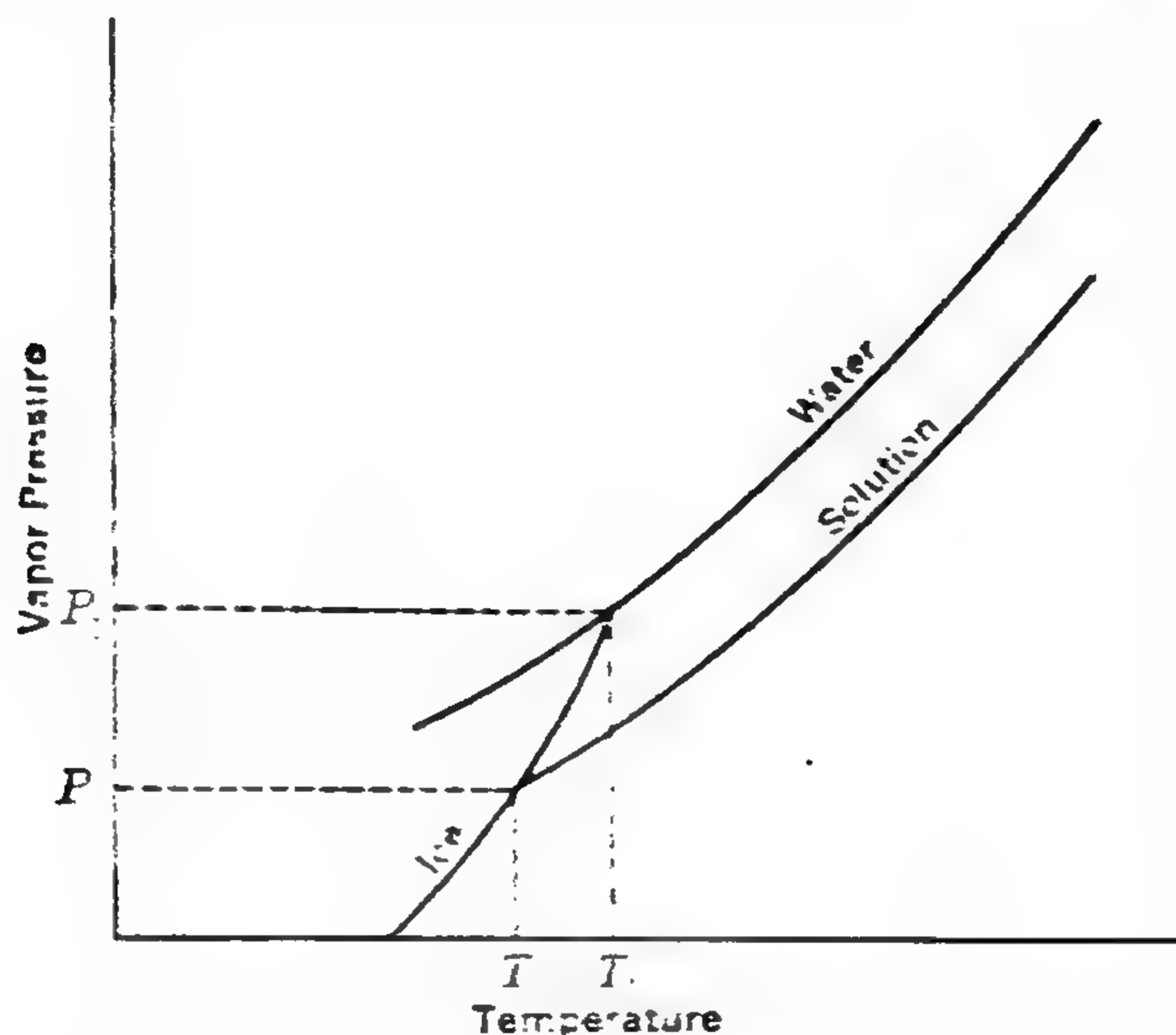


FIG. 1. Relation between the vapor-pressure lowering and freezing-point lowering of a solution.

these circumstances  $T_0 - T = P_0 - P$  will be equal to a constant. Substituting  $T_0 - T = E$  for  $P_0 - P$  in Raoult's law, we have

$$T_0 - T = EP_0 \cdot \frac{n_2}{n_1 - n_2} \quad (11)$$

where  $E$  is the proportionality constant relating freezing-point lowering and vapor-pressure lowering. For dilute solutions equation 11 reduces to

$$T_0 - T = \frac{Ex_2}{M_2} \cdot \frac{M_1}{x_1} \quad (12)$$

For purposes of molecular-weight calculations equation 12 is usually written

$$M_2 = K_f \frac{1000x_2}{\Delta T x_1} \quad (13)$$

where  $\Delta T$  is equal to  $T_0 - T$ ,  $x_1$  is the weight of the solvent,  $x_2$  the weight of the solute, and  $K_f$  is a constant whose value varies from solvent to solvent. For water it is equal to  $1.86^\circ$ . A gram of pure protein with a molecular weight of 10,000 dissolved in 100 grams of pure water would give a freezing-point lowering of

$$\Delta T = \frac{1.86 \times 1000 \times 1}{10,000 \times 100} \quad (14)$$



Quite evidently freezing-point depressions cannot be used to determine the molecular weights of high-molecular-weight compounds.

### OSMOTIC PRESSURE

If a solution is separated from the pure solvent by a membrane that is permeable to the solvent but impermeable to the solute, the solvent will diffuse into the solution. This diffusion can be prevented by imposing a counter pressure on the solution. The exact pressure required to prevent this diffusion is known as the osmotic pressure. This phenomenon is another instance of the contribution of the biological sciences to physical chemistry; the first osmotic-pressure studies were made by the botanist W. F. P. Pfeffer in 1877.

Let us attempt to understand as clearly as we can the occasion for osmotic pressure. Consider Fig. 2.

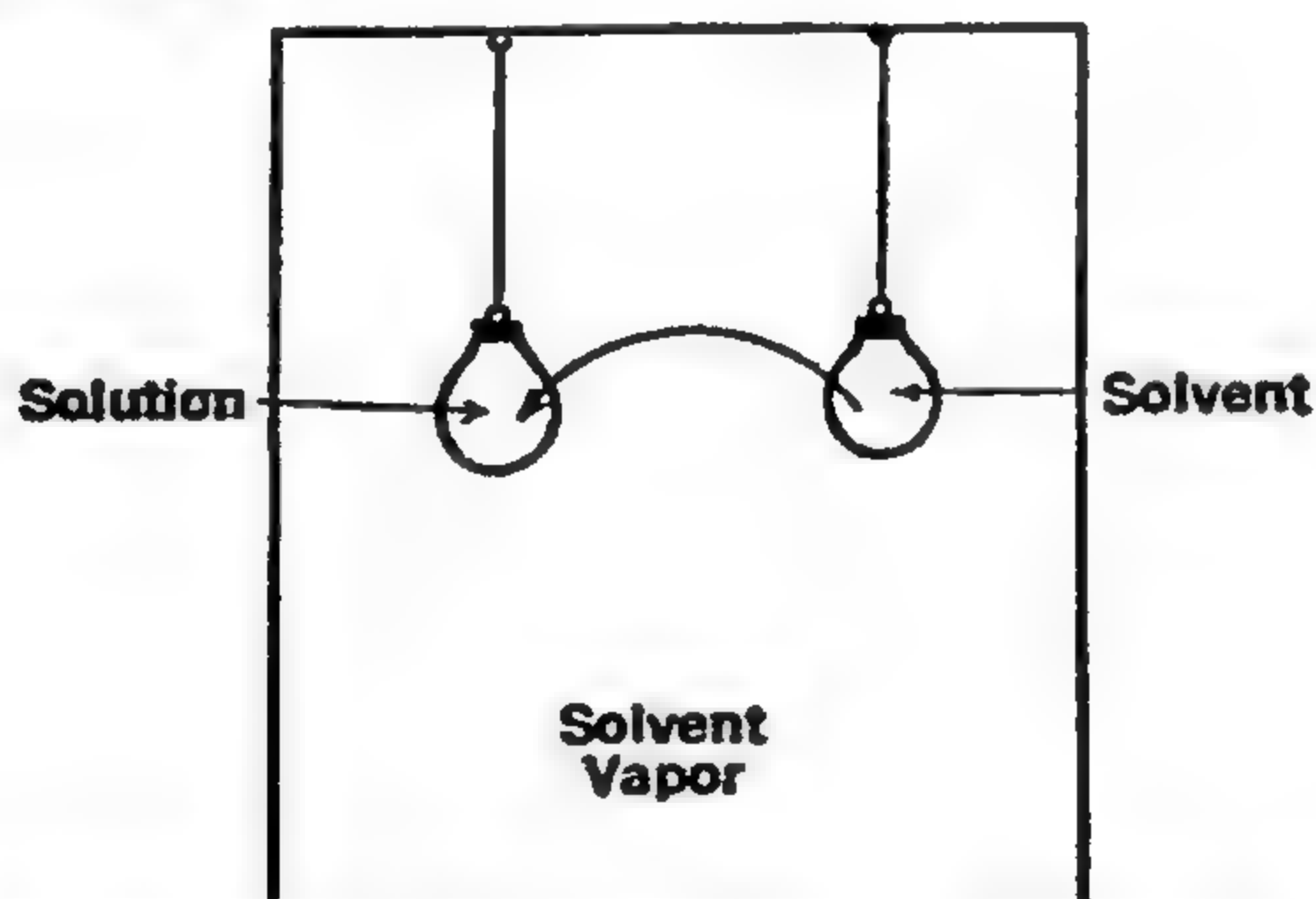


FIG. 2. Solution and solvent are inclosed in separate collodion sacks. The sacks are suspended in a sealed container, and solvent distils into the solution.

Since the vapor pressure of the solvent is greater than that of the solution, the vapor will distil from the solvent into the solution. A hydrostatic pressure inside the collodion sack containing the solution will develop as the result of this distillation. A time will be reached when the hydrostatic pressure in the solution sack prevents the further distillation of vapor from the solvent to the solution. The system is then in equilibrium. The work required at this point to transfer 1 gram-molecular weight of the solvent to the solution is  $P_h \bar{V}_1$ , where  $P_h$  is the hydrostatic pressure inside the solution sack and  $\bar{V}_1$  is the partial molar volume of the solvent. Since the system is at equilibrium and the transfer is to be conducted reversibly at constant temperature and pressure, the work done is equal to the free energy change. During this transfer we have changed 1 mole of solvent from a vapor pressure  $P_0$  to a vapor pressure  $P$ , and the free energy of this process as we have seen from Chapter 2 is

$$\Delta F = RT \ln \frac{P_0}{P} \quad (15)$$

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Equating the free energies of the two processes we have

$$P_h \bar{V}_1 = -RT \ln \frac{P}{P_0} \quad (16)$$

Substituting the value of  $P/P_0$  from equation 4, there results

$$P_h \bar{V}_1 = -RT \ln \left( 1 - \frac{n_2}{n_1 + n_2} \right) \quad (17)$$

If  $n_2$  is very small compared with  $n_1$  (dilute solution), the logarithm of  $\left( 1 - \frac{n_2}{n_1 + n_2} \right)$  is very nearly equal to  $-\frac{n_2}{n_1 + n_2}$ . Then

$$P_h \bar{V}_1 = RT \cdot \frac{n_2}{n_1 + n_2} \quad (18)$$

$P_h$  is evidently the osmotic pressure and  $\bar{V}_1$  is very nearly equal to  $M_1/\rho_1$ , where  $M_1$  is the molecular weight of the solvent and  $\rho_1$  is its density. Since  $n_2$  is assumed to be small compared to  $n_1$ , it can be neglected in the denominator of equation 18. Including all this in equation 18 and rearranging, there results

$$P_{op} = \frac{\rho_1 RT n_2}{M_1 n_1} \quad (19)$$

where  $P_{op}$  is the osmotic pressure of the solution. Since  $M_1 n_1$  equals the total weight of the solvent present in the solution, we have

$$P_{op} = \frac{\rho_1 RT n_2}{w_1} \quad (20)$$

Since  $n_2$  is the number of moles of the solute dissolved in weight  $w_1$  of the solvent, if we select  $w_1$  equal to 1 gram, equation 20 becomes

$$P_{op} = \frac{\rho_1 RTC}{M_2} \quad (21)$$

where  $C$  is the number of grams of the solute dissolved in 1 gram of solvent and  $M_2$  is the molecular weight of the solute ( $n_2$  is equal to  $C/M_2$ ).

If the osmotic pressure is expressed in centimeters of water,  $R$  has the value

$$R = \frac{22,414 \times 76.0 \times 13.597}{273.1} \quad (22)$$

$$= 8.48 \times 10^4 \text{ cc. cm. H}_2\text{O per mole per degree} \quad (22a)$$

where 22,414 is the molecular volume at 76.0 cm. of mercury pressure and 13.597 is the density of mercury at 0° C. Substituting numerical values in equation 21, we have at 25° C. the working equation

$$P_{op} = \frac{2.528 \times 10^7 \rho_1 C}{M_2} \quad (23)$$

If 0.01 gram of protein, whose molecular weight is 10,000, is dissolved in 1 gram of water at 25° C.,

$$\begin{aligned} P_{op} &= \frac{2.528 \times 10^7 \times 0.9970 \times 0.01}{10,000} \\ &= 25.20 \text{ cm. H}_2\text{O} \end{aligned} \quad (24)$$

A high-molecular-weight compound can evidently yield an osmotic pressure which is appreciable and can be conveniently measured. From calculations that we have made we see that 25.20 cm. H<sub>2</sub>O osmotic pressure is equivalent to a freezing-point depression of 0.00186° C. and to a vapor-pressure lowering of 0.00058 cm. H<sub>2</sub>O. Of these three methods only the osmotic pressure is suited to the determination of the molecular weight of proteins.

#### METHODS FOR OSMOTIC-PRESSURE MEASUREMENTS

Osmotic-pressure measurements are not conducted in the indirect manner shown in Fig. 2. Actually the solution is placed in a sack made of collodion or some other suitable substance, and the sack is immersed in the solution. The pressure developed inside the sack is measured by an appropriate manometer. The principle, however, is the same: solvent molecules distil from the solvent into the solution, causing a pressure to develop inside the sack. At equilibrium this pressure is equal to the osmotic pressure.

Osmotic-pressure measurements, as we have noted, are particularly suited to the investigation of high-molecular-weight compounds. Membranes can easily be found that will permit passage of water and of buffer molecules as well as inorganic ions but will not allow protein molecules to pass through. If the molecular weight of the protein is not too great, a reasonably dilute solution will yield an osmotic pressure which can be measured with considerable accuracy.

Several arrangements have been used to measure osmotic pressure. Among these may be mentioned the method of Burk and Greenberg<sup>5</sup> of Pauli and Fent,<sup>6</sup> of Oakley,<sup>7</sup> and of Bourdillon.<sup>8</sup> Güntelberg and Linder-

<sup>5</sup> N. F. Burk and D. M. Greenberg, *J. Biol. Chem.* 87, 197 (1930).

<sup>6</sup> Wo. Pauli and P. Fent, *Kolloid-Z.* 67, 288 (1934).

<sup>7</sup> H. B. Oakley, *Trans. Faraday Soc.* 31, 136 (1935).

<sup>8</sup> J. Bourdillon, *J. Biol. Chem.* 127, 617 (1939).



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ström-Lang<sup>9</sup> have described an arrangement that permits the measurement of the osmotic pressure with great accuracy. Shown in Fig. 3 is an apparatus that has proved useful.<sup>10</sup>

The apparatus is filled, as shown in Fig. 3, and set in a water bath at

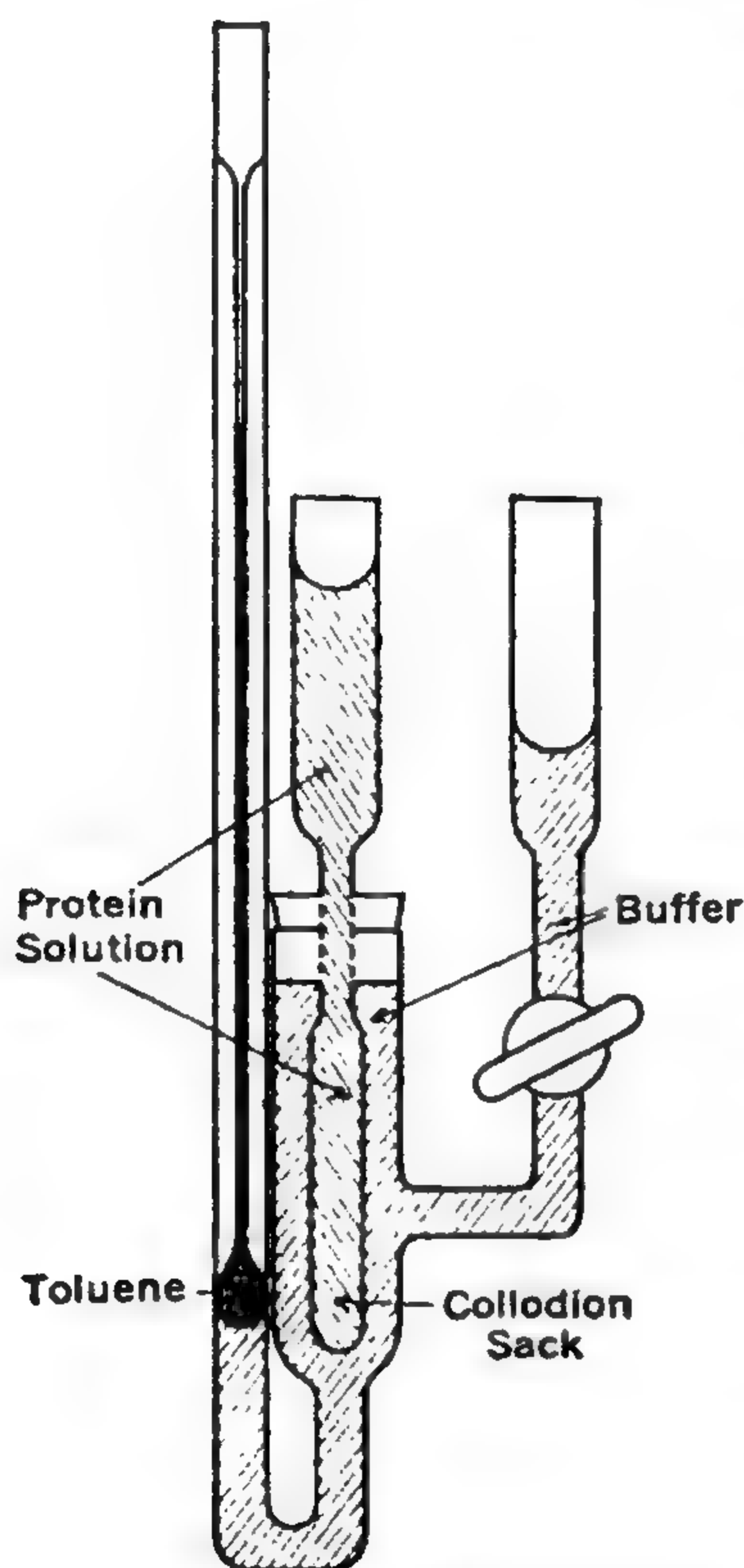


FIG. 3. Osmotic-pressure apparatus.

constant temperature. The stopcock is open, and the levels of the buffer, of the protein solution, and of the toluene in the capillary are determined with a cathetometer. The stopcock is closed, and the buffer moves into the protein solution in response to the vapor-pressure difference between the solvent and the solution. This movement forces the level of the toluene capillary down until the hydrostatic pressure developed prevents the further distillation of the solvent into the solution. The level of the toluene capillary is then read with the cathetometer, and the osmotic pressure is calculated as being equal to the difference in level between the buffer and protein solution multiplied by the density of the protein solution. To this is added the difference between the initial and equilibrium levels of the toluene multiplied by the density of the toluene. As the capillary bore is about 0.2 mm. in diameter, the amount of solvent transferred from the buffer to the protein solution is so small that it produces no significant change in protein concentration.

The above methods are what might be called static, and the time required to complete a measurement is, even in the most sensitive, appreciable. Montanna and Jilk<sup>11</sup> devised a dynamic method which consists in measuring the rate of flow of the solvent through the membrane as a result of application of an external pressure to the solution side of the membrane. The rate of flow is then plotted as a function of the external pressure, and the line is extrapolated to zero rate of flow. The intercept on the pressure

<sup>9</sup> A. V. Güntelberg and K. Linderström-Lang, *Comp. rend. trav. lab. Carlsberg* 27, No. 1 (1949).

<sup>10</sup> H. B. Bull, *J. Biol. Chem.* 137, 143 (1941).

H. B. Bull and B. T. Currie, *J. Am. Chem. Soc.* 68, 742 (1946).

<sup>11</sup> R. E. Montanna and L. T. Jilk, *J. Phys. Chem.* 45, 1374 (1941).

axis is equal to the osmotic pressure. Fuoss and Mead<sup>12</sup> have made use of this principle and have described an osmometer that has found wide favor in work with synthetic high polymers, although it has not been used to determine the osmotic pressure of protein solutions. The method is to observe the displacement of the meniscus in a vertical capillary as a function of time when the external pressure exceeds the osmotic pressure and again when the external pressure is less than the osmotic pressure. Readings of the moving meniscus are made at 30-sec. intervals for 6 to 8 min. The half-sum of the rising and falling curves is then plotted against time; since the curves were started approximately the same distance from equilibrium, but from opposite sides, the half-sum differs from their mutual asymptote only by small second-order terms. In practice, the half-sum is constant within several minutes and determines the desired asymptote directly. Figure 4 illustrates the type of curves observed. The dynamic method requires that the membrane be well supported.

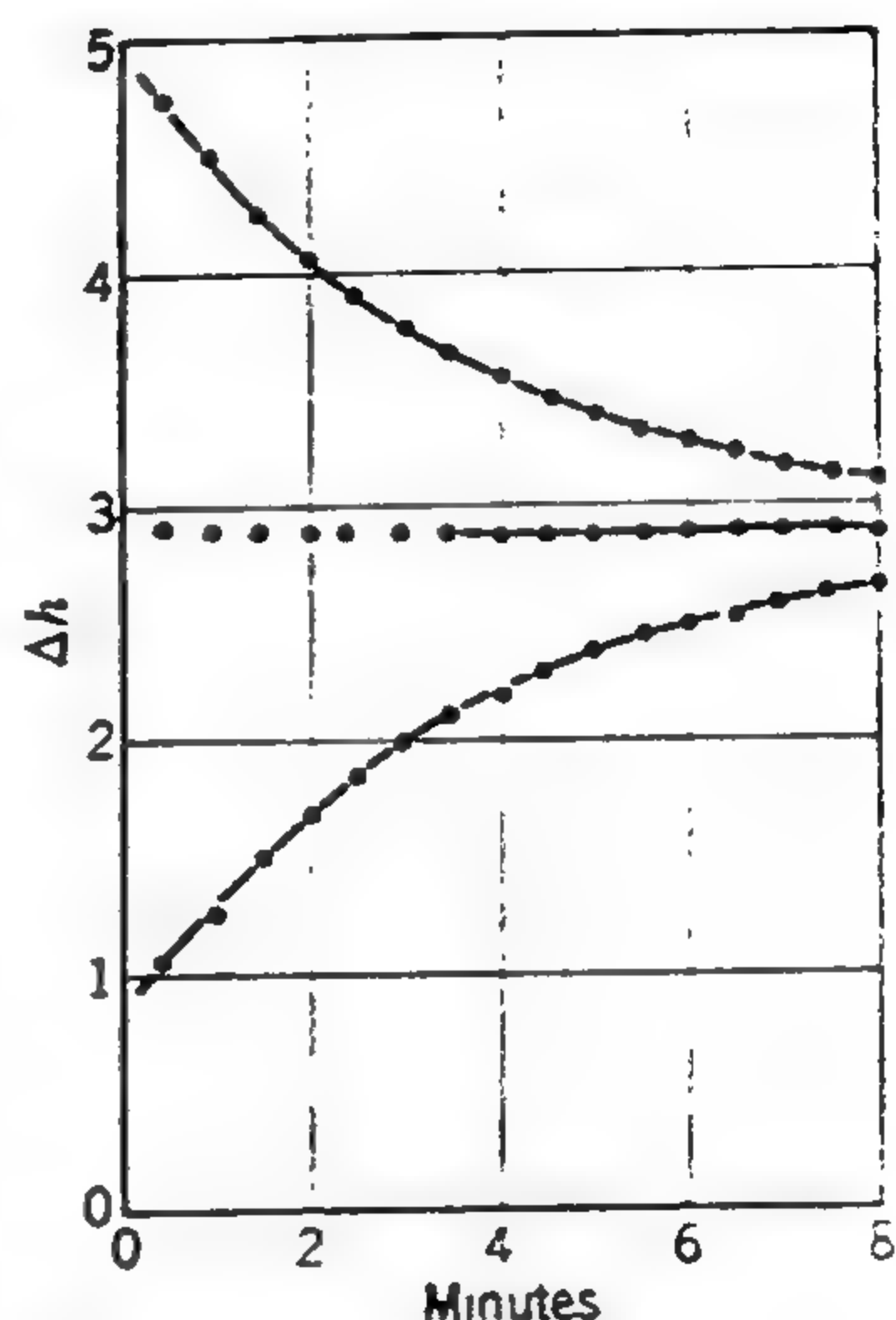


FIG. 4. Rate curves for osmometer with vertical capillaries. (Fuoss and Mead.)

### RESULTS OF OSMOTIC-PRESSURE MEASUREMENTS

In the early work on protein osmotic pressure, confusing results were obtained, and the molecular weights calculated from these results were very discordant. The difficulty arose from a number of sources. Among these may be mentioned: (1) inadequate experimental technique, (2) failure to appreciate the importance of the electrical charges on the protein molecules, (3) incorrect manner of expressing protein concentrations, and (4) impure protein preparations.

For the purposes of calculating the molecular weights of proteins from osmotic pressure, the conditions for measurement and calculations are exacting. Either the experimental arrangement must be so designed that the capillary rise of the liquid due to surface tension cancels out of the calculation, or the capillary rise must be corrected for. The time of attainment of equilibrium cannot be too long or the protein will be subject to decomposition. The measurement must be made in the presence of suffi-

<sup>12</sup> R. M. Fuoss and D. J. Mead, *J. Phys. Chem.* 47, 59 (1943).

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cient electrolyte to wipe out all electrical effects due to the electrostatic charges on the protein molecules. The degree of purity of the protein cannot be overemphasized. Only well-defined, highly purified proteins should be used. If impure proteins are studied, the experimenter is wasting his time.

In Fig. 5 is shown the osmotic pressure of egg albumin and of serum globulin in urea solutions<sup>5</sup> as a function of protein concentration.

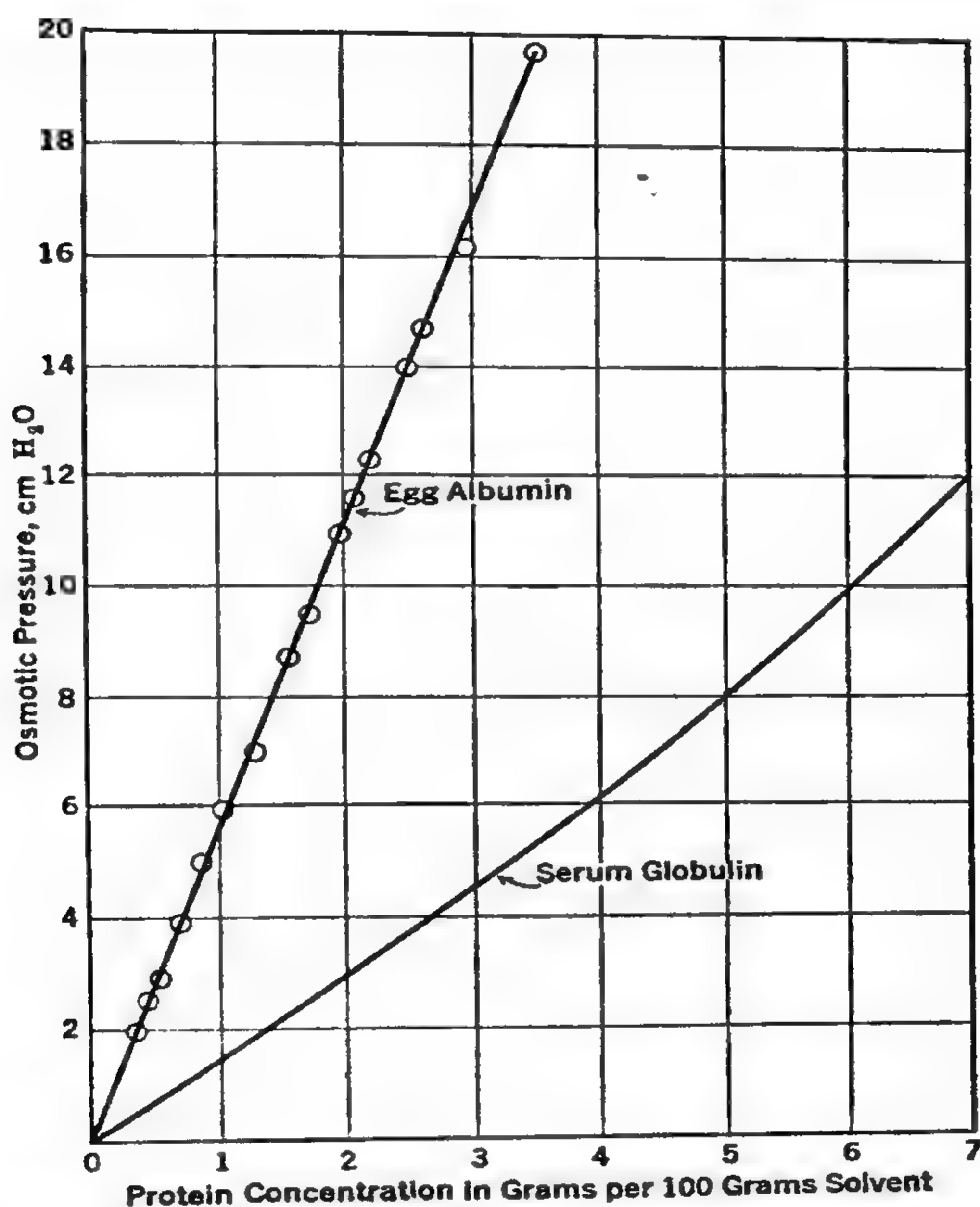


FIG. 5. Osmotic pressures of egg albumin (Bull) and of serum globulin (Burk and Greenberg) as functions of protein concentration at 25° C.

Note that over the concentrations covered the relation between osmotic pressure and the concentration of egg albumin is a linear one as demanded by the simple theory outlined above. A number of proteins, however, show a departure from a linear relation at quite low concentrations. Serum globulin shown in Fig. 5 is an example of such a protein. The osmotic



pressure can be expressed in terms of an expansion series of concentration terms.<sup>13</sup>

$$P_{0p} = \rho RT \left( \frac{C}{M_2} + A_2 C^2 + A_3 C^3 + \dots \right) \quad (25)$$

If  $P_{0p}/C$  be plotted against  $C$  and the line be extrapolated to zero concentration, the value of the intercept is  $\rho_1 RT/M_2$ , from which  $M_2$  may be calculated. It is intriguing, however, to inquire into the reason or reasons for the departure of the  $P_{0p}$ - $C$  plots from linearity.

For any isothermal process at constant pressure, we have (see Chapter 2)

$$\Delta F = \Delta H - T \Delta S \quad (26)$$

Since we are dealing with partial molal quantities when the solvent is transferred across the membrane, we write

$$\overline{\Delta F} = \overline{\Delta H} - T \overline{\Delta S} \quad (27)$$

and, since  $\overline{\Delta F}$  is equal to  $-P_{0p}\overline{V}_1$ , we have

$$P_{0p} = \frac{T \overline{\Delta S}}{\overline{V}_1} - \frac{\overline{\Delta H}_1}{\overline{V}_1} \quad (28)$$

In short, the osmotic pressure is made up of an entropy term and a heat term, and the deviation of the osmotic pressure from ideality might involve either or both of these terms. If the heat of solution is positive (heat absorbed) during the transfer of water from the solvent to the solution, the osmotic pressure will be less than that calculated; if it is negative, the osmotic pressure will be greater. If the partial molal heat is zero, it approaches zero as the solution becomes infinitely dilute.

$$P_{0p} = \frac{T \overline{\Delta S}}{\overline{V}_1} \quad (29)$$

The partial molal entropy is really the entropy of mixing; this factor has been investigated by Flory,<sup>14</sup> by Huggins,<sup>15</sup> and by Zimm,<sup>16</sup> using the methods of statistical mechanics. The entropy of mixing is related to the number of ways in which molecules can be arranged in solution; for spherical molecules of equal size, where there is no interaction, the entropy is

$$\Delta S = -k(n' \ln N_1 + n'' \ln N_2) \quad (30)$$

where  $k$  is Boltzmann's constant,  $n'$  is the number of solvent molecules,

<sup>13</sup> W. G. McMillan and J. E. Mayer, *J. Chem. Phys.* 13, 276 (1945).

<sup>14</sup> P. J. Flory, *J. Chem. Phys.* 12, 425 (1944).

<sup>15</sup> M. L. Huggins, *J. Phys. & Colloid Chem.* 52, 248 (1948).

<sup>16</sup> B. H. Zimm, *J. Chem. Phys.* 14, 164 (1946).

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$N_1$  is the mole fraction of the solvent,  $n''$  is the number of solute molecules, and  $N_2$  is the mole fraction of the solute. For a dilute solution and for one mole of solvent equation 30 reduces to

$$\Delta S = -R \ln N_1 \quad (30a)$$

It can be seen that, if equation 30a be substituted in equation 29, we shall arrive directly at equation 17; equation 30a is really an expression of Raoult's law. For systems whose solute molecules are not of the same size as the solvent molecules, for asymmetric solute molecules with and without flexibility there will be a departure from random mixing. What we are immediately interested in is the effect of particle size and shape on the coefficient  $A_2$  in equation 25 (heat of mixing to be considered zero). Zimm has calculated  $A_2$  for large spherical solute molecules, as well as for long rigid rods. For rigid spherical solute molecules Zimm finds  $A_2$  equal to  $2\pi a^3 N_0 / 3M_2^2$ , and for long rigid rod-like solute molecules  $A_2$  is  $\pi N_0 d l^2 / 4M_2^2$ , where  $N_0$  is Avogadro's number,  $a$  is the diameter of the sphere,  $d$  is the cross-sectional diameter of the rigid rod-like molecules, and  $l$  is its length. The dimensions are to be expressed in centimeters, and  $M_2$  is the molecular weight of the solute molecule.

The ratio of the observed osmotic pressure of a solution of spheres to that calculated from Raoult's law is then

$$\text{Ratio} = \left( \frac{RTC}{M_2} + A_2 RTC^2 \right) \frac{M_2}{RTC} \quad (31)$$

$$= 1 + A_2 M_2 C \quad (32)$$

Consider, for example, egg albumin whose molecular weight is 45,000 and whose partial specific volume is 0.75 and let us assume that the egg albumin molecules are spherical. Under these conditions  $A_2$  is  $6.7 \times 10^{-5}$ , and, accordingly,

$$\text{Ratio} = 1 + 6.7 \times 10^{-5} M_2 C \quad (33)$$

and, since  $M_2$  is 45,000,

$$\text{Ratio} = 1 + 3C \quad (34)$$

where  $C$  is expressed in grams of protein per cubic centimeter of solution. It appears that the entropy term in a 1 per cent egg albumin solution will increase the osmotic pressure about 3 per cent. A 10 per cent solution should show a departure corresponding to about 30 per cent increase in osmotic pressure. If the concentration had been expressed in grams of protein per gram of solvent, the departure from ideality would have been somewhat less.

If rod-like molecules are made flexible by introducing universal joints at intervals along the rods and if the segments between the joints are still long enough to be treated as rods themselves,  $A_2$  is  $\pi N_0 n^2 \lambda^2 / 4M_2^2$ , where  $\lambda$  is the length of the segment and  $n$  is the number of segments. Notice that this value is essentially the same as that for rigid rods. The conclusion is reached, therefore, that the introduction of a moderate number of joints in a stiff molecule has no appreciable effect on  $A_2$ . The joints in practically all high biopolymers are stiff, and, accordingly,  $A_2$  would probably be very close to that for rigid rods (tobacco mosaic virus protein, etc.).

The influence of heat of mixing has been neglected as well as entropy terms arising from interaction between solute molecules. It is difficult if not impossible to predict the extent of these corrections. As the solution becomes more and more dilute they become less and less important. Another factor that may be involved is the solvation of the solute molecules, i.e., in a loose structure the solvent may penetrate between the polymer chains. When solvent transfer is made across the membrane, the solvent associated with the solute is not available for transfer. This has the effect of making the solution more concentrated in respect to the solvent. This effect has been discussed by Güntelberg and Linderström-Lang.<sup>9</sup> These authors likewise discuss association between protein molecules.

It can be seen that the factors that determine the magnitude of  $A_2$  in equation 25 are fairly complex and not always predictable. A thorough-going experimental study of the variation of  $A_2$  in protein and other high biopolymeric solutions is badly needed.

## DONNAN EQUILIBRIUM

In addition to the entropy and heat effects discussed above, electrostatic charges on the high biopolymers will cause departure from ideality. In 1911 Donnan<sup>11</sup> published his well-known theory of membrane equilibria which takes into account the influence of electrostatic charges. A Donnan equilibrium arises whenever a charged particle is constrained in its movements. The classical constraint is a membrane through which a charged particle cannot pass. Suppose we titrate a protein with sodium hydroxide to produce the sodium salt of the protein. We then add a certain amount of sodium chloride and separate this solution by means of a membrane, such as cellophane, from a solution containing sodium chloride of the same concentration as the protein solution. This situation is shown diagrammatically in Fig. 6A and Fig. 6B.

In order to attain the equilibrium state (Fig. 6B), NaCl has diffused from compartment I to compartment II. This diffusion has resulted from the concentration gradient of  $\text{Na}^+$ . In order to maintain electrical neutrality

<sup>11</sup> F. G. Donnan, *Z. Elektrochem.* 17, 572 (1911).



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an equivalent amount of  $\text{Cl}^-$  must diffuse along with the  $\text{Na}^+$ . This process continues until the diffusion tendency of  $\text{Na}^+$  from I to II equals the diffusion tendency of  $\text{Cl}^-$  in the opposite direction. At this point the equilibrium state is reached. Let us consider the quantitative aspects of these transfers.

$\text{P}^- = C_1$ $\text{Na}^+ = C_1 + C_2$ $\text{Cl}^- = C_2$ $v_1$ <b>I</b>	$\text{Na}^+ = C_2$ $\text{Cl}^- = C_2$ $v_2$ <b>II</b>
---	--

FIG. 6A. Initial conditions leading to a Donnan equilibrium. Dotted line represents a membrane permeable to all components except the protein ion ( $\text{P}^-$ ).

$\text{P}^- = C_1$ $\text{Na}^+ = C_1 + C_2 - x/v_1$ $\text{Cl}^- = C_2 - x/v_1$ $v_1$ <b>I</b>	$\text{Na}^+ = C_2 + x/v_2$ $\text{Cl}^- = C_2 + x/v_2$ $v_2$ <b>II</b>
---	--

FIG. 6B. Equilibrium conditions.  $x$  moles of  $\text{NaCl}$  have been transferred from compartment I to compartment II.

The free energy required to transfer one mole of  $\text{Cl}^-$  from compartment II to compartment I in a reversible manner at constant temperature and pressure is

$$\Delta F = RT \ln \frac{\text{Cl}_1^-}{\text{Cl}_2^-} \quad (35)$$

and, similarly, the free energy required to transfer one mole of  $\text{Na}^+$  is

$$\Delta F = RT \ln \frac{\text{Na}_1^+}{\text{Na}_2^+} \quad (36)$$

where the subscripts 1 and 2 denote compartments I and II, respectively. At equilibrium, the total free-energy change must be zero and

$$RT \ln \frac{\text{Cl}_1^-}{\text{Cl}_2^-} + RT \ln \frac{\text{Na}_1^+}{\text{Na}_2^+} = 0 \quad (37)$$

from which

$$\frac{\text{Cl}_2^-}{\text{Cl}_1^-} = \frac{\text{Na}_1^+}{\text{Na}_2^+} = r \quad (38)$$

The ratio of the concentration in compartment I to that in compartment II of any univalent anion must equal the ratio of the concentration in II to that in I of any univalent cation. This statement is perfectly general and can be proved for any pair of univalent ions in precisely the same way as we have for  $\text{Na}^+$  and  $\text{Cl}^-$ . If polyvalent cations and anions are introduced, the relation has to be extended, and we have that for any pair of anions and cations

$$\left(\frac{A_2}{A_1}\right)^{1/z_a} = \left(\frac{M_1}{M_2}\right)^{1/z_m} = r \quad (39)$$

where  $A$  and  $M$  represent anions and cations and  $z_a$  and  $z_m$  are their respective valences.

Referring to the simple situation depicted in Fig. 6B, we have at equilibrium

$$r = \frac{\text{Cl}_2^-}{\text{Cl}_1^-} = \frac{C_2 + x/v_2}{C_2 - x/v_1} = \frac{\text{Na}_1^+}{\text{Na}_2^+} = \frac{C_1 + C_2 - x/v_1}{C_2 + x/v_2} \quad (40)$$

If  $v_1$  and  $v_2$  are both equal, we can easily eliminate  $x$  from equation 40 to obtain

$$r = 1 + \frac{C_1}{2C_2} \quad (41)$$

$C_1$  is evidently the equivalent concentration of the protein and is equal to  $Wz/M_2v_1$ , where  $W$  is the weight of the protein,  $z$  is the effective valence of the protein, and  $M_2$  is the molecular weight of the protein. Equation 41 then becomes

$$r = 1 + \frac{Wz}{2M_2C_2v_1} \quad (42)$$

We can conclude from equation 42 that, the greater the equivalent concentration of protein (the greater the non-diffusible charge), the more uneven will be the final distribution of electrolyte between the two compartments (the greater the value of  $r$ ). On the other hand, if the initial salt

TABLE 1  
RATIO OF  $(\text{NaCl})_2$  TO  $(\text{NaCl})_1$  AT EQUILIBRIUM FOR AN  
EQUIVALENT PROTEIN CONCENTRATION OF 0.1

Initial NaCl Concentration	$r$
0.01	6.03
0.05	2.00
0.10	1.50
1.00	1.05

concentration is high, the distribution of electrolyte will approach unity at equilibrium and the Donnan equilibrium distribution will be of no practical importance. For example, if the equivalent concentration of protein is 0.1 and we add varying concentrations of NaCl, the value of  $r$  calculated from equation 42 ( $r_1$  equals  $r_2$ ) is given in Table 1.

Owing to the uneven distribution of ions in a Donnan equilibrium, there must exist a potential difference between the two compartments. This potential, in the example we have been considering (Fig. 6), will be

$$E = \frac{RT}{F} \ln \frac{[\text{Cl}^-]_1}{[\text{Cl}^-]_2} \quad (43)$$

The potential arises from the fact that the concentration of  $\text{Cl}^-$  in compartment II is greater than that in compartment I. The equalization of this ionic concentration difference is prevented by the requirement for electrical neutrality. If, however, reversible electrodes are inserted into the two compartments and the electrodes connected through an external circuit, a mechanism is provided whereby cations can flow into compartment II and out of compartment I. This enables the  $\text{Cl}^-$  to migrate from compartment II to compartment I in response to its concentration gradient, and a current flows through the external circuit; electrical neutrality is thereby maintained. It must not be thought that, if the current is allowed to flow and the electrodes removed, the same Donnan equilibrium will appear as was originally present. If this were true, an arrangement could be made which would function as a perpetual-motion machine. Remember that ions have flowed into and out of the two electrodes and the situation after current flow is quite different from the original one. A peculiarity is that the electrodes used to measure the potential must not be capable of producing an ion which is involved in the Donnan equilibrium. If such an electrode is used, the potential across the two compartments will always appear to be zero. Hydrogen electrodes, accordingly, cannot be used because water always contains hydrogen ions which distribute themselves according to the Donnan equilibrium. Suitable electrodes are calomel half-cells connected by KCl salt bridges to the compartments.

We have not discussed the situation that arises when acids or bases are added to compartment II instead of neutral salt. The introduction of acids or of bases will evidently complicate the distribution because they will alter the charge on the protein and change its equivalent concentration. The situation can be analyzed, however, and it will be found at equilibrium that

$$\frac{[\text{H}^+]_1}{[\text{H}^+]_2} = \frac{[\text{OH}^-]_2}{[\text{OH}^-]_1} = \frac{[\text{Cl}^-]_2}{[\text{Cl}^-]_1} = \frac{[\text{Na}^+]_1}{[\text{Na}^+]_2} \quad (44)$$



If the protein is acid to its isoelectric point, solution in compartment II will be more acid than that in compartment I; and if the protein is basic to its isoelectric point, compartment II will be more basic than compartment I.

The presence of Donnan equilibrium has important consequences for the osmotic pressure of a system. In general, the osmotic-pressure difference between the first and the second compartment would be expected to be

$$P_{0p} = RT(\Sigma C_1 - \Sigma C_2) \quad (45)$$

where  $\Sigma C_1$  is the total molal concentration of dissolved material in compartment I and  $\Sigma C_2$  that in compartment II. It is quite clear that a Donnan equilibrium will always increase the osmotic pressure of a protein solution above that expected from the protein alone. The osmotic pressure due to the diffusible ions alone is for the situation in Fig. 6B

$$P_E = RT(\text{Na}_1^+ + \text{Cl}_1^- - \text{Na}_2^+ - \text{Cl}_2^-) \quad (46)$$

Substituting the concentrations of the various ions shown in Fig. 6B in equation 46, we obtain a very complicated expression if  $\tau_1$  is not equal to  $\tau_2$ . If we set  $\tau_1$  equal to  $\tau_2$ , this complicated expression reduces to

$$P_E = RTC_1 \left( 1 - \frac{4C_2}{4C_2 + C_1} \right) \quad (47)$$

Since  $C_1$  is equal to  $Wz/M_2\tau_1$  we have, substituting this value for  $C_1$  in equation 47,

$$P_E = \frac{RTWz}{M_2\tau_1} \left( 1 - \frac{4C_2}{4C_2 + Wz/M_2\tau_1} \right) \quad (48)$$

from which the additional osmotic pressure due to the uneven distribution of ions can be calculated. To obtain the total osmotic pressure, the osmotic pressure due to the protein alone and obtained from equation 21 has to be added.

Scatchard, Batchelder, and Brown<sup>15</sup> have carried out a comprehensive series of measurements on the osmotic pressure and membrane potentials of solutions of bovine serum albumin in the presence of sodium chloride at different pH values. They found evidence of binding of salt ions by the albumin.

<sup>15</sup> G. Scatchard, A. C. Batchelder, and A. Brown, *J. Am. Chem. Soc.* **68**, 2320 (1946).

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### DONNAN EQUILIBRIUM AND OSMOTIC PRESSURE BETWEEN PARALLEL PLATES

If we consider  $\tau_2$  to be very much larger than  $\tau_1$ , we obtain from equation 40

$$r = \frac{C_1 + C_2 - (x/\tau_1)}{C_2} = \frac{C_2}{C_2 - (x/\tau_1)} \quad (49)$$

and, rearranging equation 49, there results

$$\left(C_1 + C_2 - \frac{x}{\tau_1}\right)\left(C_2 - \frac{x}{\tau_1}\right) = C_2^2 \quad (50)$$

Now under the conditions where  $\tau_2 \gg \tau_1$  it can be shown that

$$P_E = RT \left( C_1 - \frac{2x}{\tau_1} \right) \quad (51)$$

Combining equations 51, 48, and 49 and solving for  $P_E$ , we have

$$P_E = 2RT[(4C_2^2 + C_1^2)^{1/2} - 2C_2] \quad (52)$$

Suppose that we cover two glass plates with a monolayer of egg albumin. These plates are each 1 cm. square. They are immersed in 0.01 *M* NaCl, and their faces are brought together in an exactly parallel position. The problem is to calculate the osmotic pressure between the plates as a function of the distance apart. This osmotic pressure would manifest itself as a force of repulsion.

The amount of egg albumin on each of the inner faces of the parallel plates would be approximately  $1.25 \times 10^{-7}$  gram and the molecular weight of egg albumin is about 45,000; accordingly, this weight of egg albumin would correspond to about  $2.8 \times 10^{-12}$  mole of protein. Suppose that at the pH of the solution each molecule of protein has 10 negative charges; then the total number of equivalents on the two inner faces of the glass plates would be  $5.56 \times 10^{-11}$ . At 25° C., and expressing the osmotic pressure in centimeters of water, equation 52 then becomes

$$P_E = 5.06 \times 10^7 \left[ \left( 4 \times 10^{-10} + \frac{30.9 \times 10^{-22}}{d^2} \right)^{1/2} - 2 \times 10^{-6} \right] \quad (53)$$

where  $d$  is the distance separating the plates in centimeters. Figure 7 shows the force of repulsion between the plates as a function of the distance of separation. The calculated forces and distances are of such magnitude that it should be possible to design experiments to test this theory. Shown also in Fig. 7 is the force of repulsion calculated by means of the theory of Verwey and Overbeek (Chapter 9).

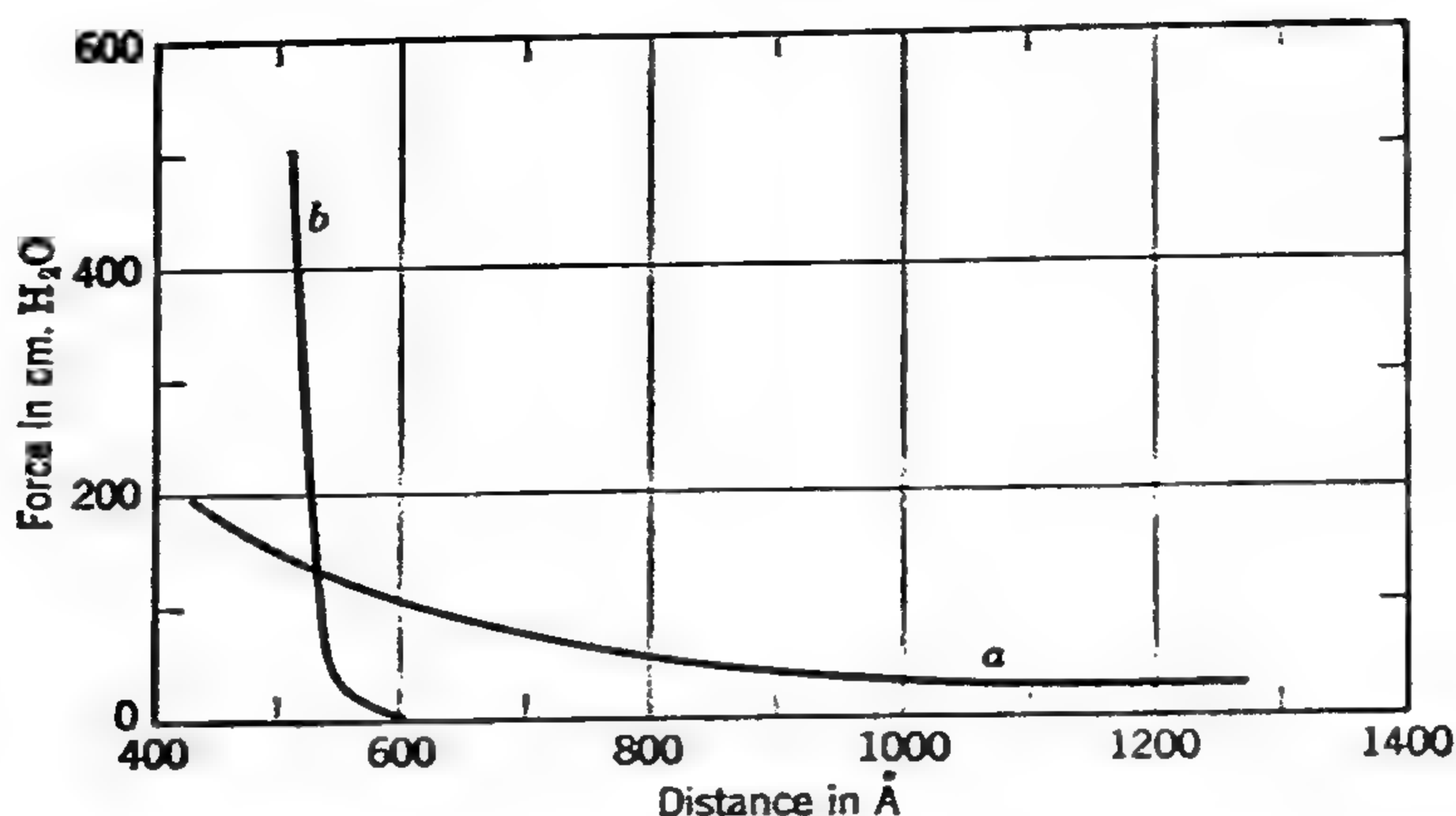


FIG. 7. Force of repulsion between two charged plates 1 sq. centimeter in area immersed in 0.01 *M* NaCl. (a) As calculated from Donnan equilibrium. (b) As calculated by the equation of Verwey and Overbeek.

## LIGHT SCATTER

It is a common observation that a beam of light becomes visible when passed through a smoky atmosphere; the light is scattered from the smoke particles, and this scattering is known as a Tyndall cone.<sup>19</sup>

The study of light scatter has assumed considerable importance since it is possible to gather valuable information about suspended particles by means of such studies. The literature has become fairly extensive; an excellent review is that by Oster.<sup>20</sup>

The problem of light scatter by particles whose radii are small compared with the wavelength of light was first investigated by Lord Rayleigh,<sup>21</sup> who derived an equation expressing the turbidity of an ideal gas of low density. If this gas contains  $\nu$  molecules per cubic centimeter and the polarizability of a molecule is  $\alpha$ , then the turbidity  $\tau$  of the gas due to scattering can be calculated by the formula

$$\tau = \frac{8\pi}{3} \left( \frac{2\pi}{\lambda} \right)^4 \nu \alpha^2 \quad (54)$$

where  $\lambda$  is the wavelength of light. The turbidity is also called the attenuation or extinction coefficient due to scattering and is given by the relation

$$\tau = \frac{1}{l} \ln \frac{I_0}{I} \quad (55)$$

where  $l$  is the distance through the gas,  $I_0$  is the intensity of the incident

<sup>19</sup> J. Tyndall, *Phil. Mag.* 37, 384 (1869).

<sup>20</sup> G. Oster, *Chem. Revs.* 43, 319 (1948).

<sup>21</sup> Lord Rayleigh, *Phil. Mag.* 41, 447 (1871).



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light, and  $I$  is that of the transmitted light. In effect, Lord Rayleigh considered that the particles act as linear electric oscillators and that the oscillating electric field of light incident upon the particles induces an oscillating electric moment in the particle the energy of which appears as scattered light of the same wavelength as the incident light. The polarizability  $\alpha$  is the electric moment induced in the molecule by an electric field of unit strength. The polarizability is connected with the index of refraction by the relation

$$\alpha = \frac{n - 1}{2\pi\nu} \quad (56)$$

where  $n$  is the index of refraction. Then, combining equations 54 and 56, we have

$$\tau = \frac{32\pi^3}{3} \frac{(n - 1)^2}{\lambda^4} \frac{1}{\nu} \quad (57)$$

Einstein<sup>22</sup> considered light scattered from a liquid to arise from local thermal fluctuations in the density of the liquid which make the medium optically inhomogeneous. He obtained the magnitude of these fluctuations by comparing this thermal energy with the work that would have to be done by an outside pressure to accomplish the same fluctuations. Such a comparison led to

$$\tau = \frac{32\pi^3}{3} \frac{1}{\lambda^4} \frac{kT}{K} \left( \frac{n \, dn}{dp} \right)^2 \quad (58)$$

where  $p$  is the hydrostatic pressure,  $k$  is Boltzmann's constant, and  $K$  is the compressibility of the liquid.

For solutions<sup>23</sup> we must, in addition to the thermal fluctuations of density, consider local differences due to fluctuations in concentration. Instead of the hydrostatic pressure we consider the osmotic pressure that would be necessary to cause such local fluctuations in concentrations. It can be shown that the fluctuations in concentration lead to a scattered intensity proportional to

$$\frac{kT}{\lambda^4} \frac{C^2 (dn^2/dC)^2}{C \, dP/dC} \quad (59)$$

where  $P$  is the osmotic pressure and  $C$  is the concentration in grams per cubic centimeter of solution. We finally arrive at the equation

$$\tau = \frac{32\pi^3}{3} \frac{n_0^3 (n - n_0)^2}{\lambda^4} \frac{1}{C \, dP/dC} \quad (60)$$

<sup>22</sup> A. Einstein, *Ann. Physik* 33, 1275 (1910).

<sup>23</sup> P. Debye, *J. Applied Phys.* 15, 338 (1944).

where  $n_0$  is the refractive index of the solvent and  $n$  is the refractive index of the solution. As we have seen, the osmotic pressure can be expressed by the equation

$$P = \frac{RTC}{M} + A_2C^2 \quad (61)$$

or differentiating equation 61 in respect to  $C$

$$\frac{dP}{dC} = \frac{RT}{M} + 2A_2C \quad (62)$$

If we let

$$\frac{1}{H} = \frac{3\lambda^4 NC^2}{32\pi^3 n_0^2 (n_0 - n)^2} \quad (63)$$

where  $N$  is Avogadro's number, and combine equations 63, 62, and 60 and rearrange, we have

$$\frac{HC}{\tau} = \frac{1}{M} + \frac{2A_2C}{RT} \quad (64)$$

Accordingly, if we plot  $HC/\tau$  against  $C$ , the slope of the line will yield  $2A_2/RT$  and the intercept at zero concentration will give the reciprocal of the molecular weight.

The intensity of the scattered light varies with the angle between the observer and the incident beam. The scattered light can be resolved into two components, the horizontally polarized and the vertically polarized; the light scattered at  $90^\circ$  will be completely vertically polarized, and the horizontal component will be zero. The intensity of the total scattered light forms an intensity envelope, the equation of which is in polar coordinates

$$\rho = 1 + \cos^2 \frac{\theta}{2} \quad (65)$$

If the radii of the particles in suspension become comparable in size with the wavelength of the incident light, the scattering becomes progressively more pronounced in the forward direction.<sup>24</sup> Figure 8 shows the distribution of the intensity of scatter for small spheres (Fig. 8a) and for spheres whose radii are nearly equal to the wavelength of the incident light (Fig. 8b).

The ratio of the scattered light in the forward direction to that in the backward direction is a function of the size of the sphere. It is also a function of the shape of the particles. Figure 9 shows a comparison of the ratio of the intensity of the light scattered at  $45^\circ$  to that scattered at  $135^\circ$  as a function of  $L/\lambda'$ .  $\lambda'$  is the wavelength of the light in the medium, and  $L$

<sup>24</sup> H. Blumer, *Z. Physik* 32, 119 (1925); 38, 304 (1926).

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is the diameter of the sphere for spherical particles.  $L$  is also the length of the rod for rod-like particles and is the root-mean-square distance between the ends of a polymer forming a random coil.

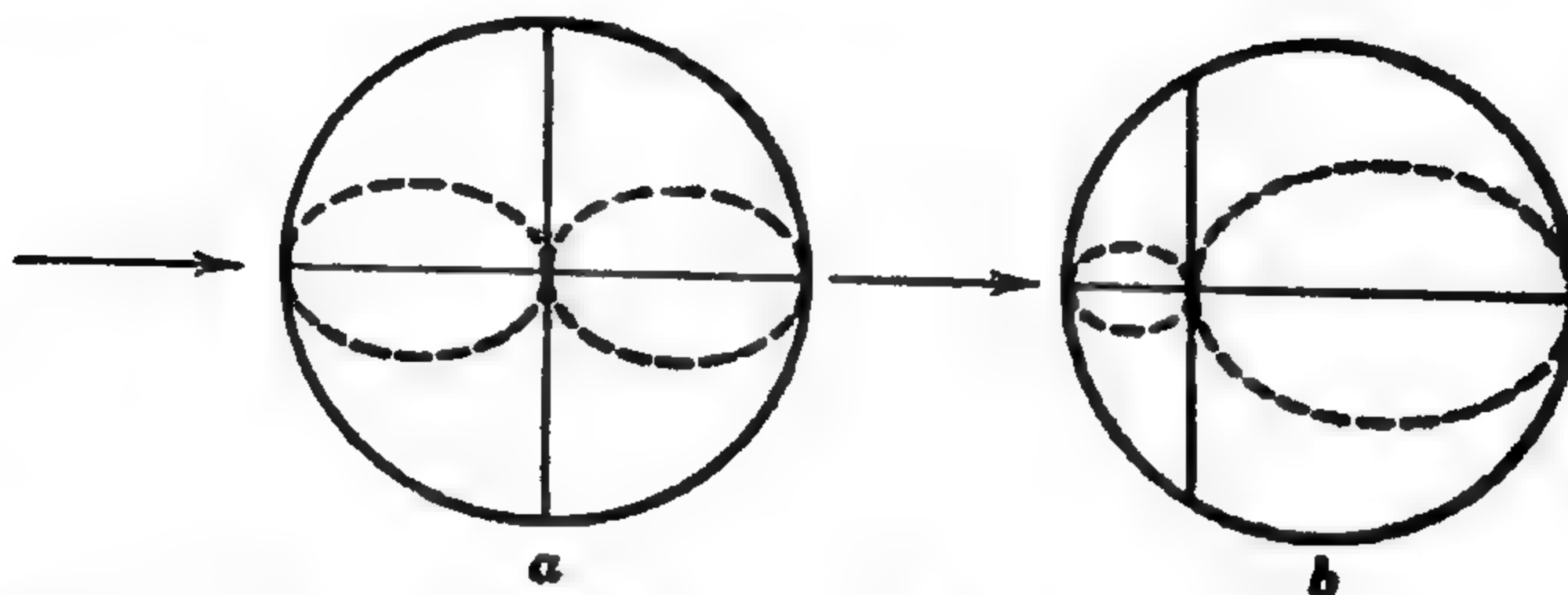


FIG. 8. Angular intensity scattering diagrams for spheres. The inner dotted curves are for horizontally polarized light, and the outer circles for vertically polarized light. (a) Particles much smaller than the wavelength of light. (b) Particles about the same size as the wavelength of light.

If the particles possess a linear dimension greater than about one-tenth the wavelength of the incident light, they cannot be considered optically small and the light scattered becomes a complicated function of wavelength and particle size. Whereas for small particles the turbidity is proportional to the sixth power of the radius and inversely proportional to the fourth power of  $\lambda$ , for particles whose radii are comparable in size to the wavelength of the incident light, the turbidity is proportional to the fourth power

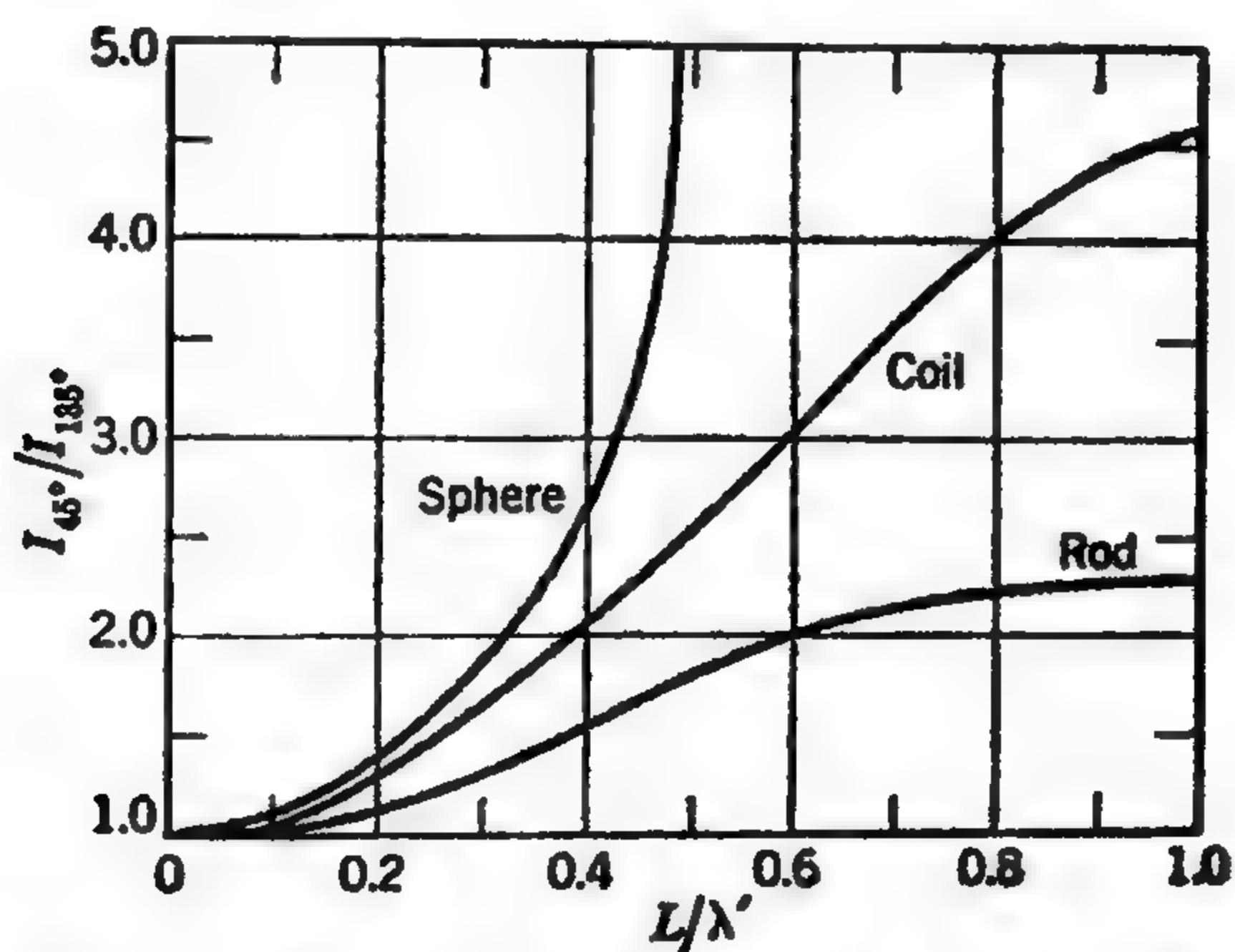


FIG. 9. Ratio of intensity of scatter at  $45^\circ$  to that at  $135^\circ$  as a function of  $L/\lambda'$  for spheres, coils, and rods of low relative index of refraction. (Oster.)

of the radius and inversely proportional to the square of  $\lambda$ . For very large particles, the turbidity is proportional to the square of the radius and is independent of the wavelength. The light scattered from suspensions of



bacteria is not critically dependent on particle size and hence offers a convenient means of determining the concentration of such a suspension.

When the particles become microscopically visible, a rainbow effect comes in which involves ordinary refraction and reflection with resulting reinforcing and cancellation of intensities at certain angles. This phenomenon has been made use of by Millar,<sup>22</sup> Poulder,<sup>23</sup> Lucke,<sup>24</sup> and others to measure the size of cells in suspension and to study their osmotic behavior. When light from a slit is passed through a cellular suspension of fairly uniform sizes, a series of interference fringes is noted and the diameter of the cells in suspension is given by

$$d \sin \theta = \frac{Z}{\pi} \lambda \quad (6)$$

where  $d$  is the diameter of the cells,  $\lambda$  is the wavelength of the light,  $\theta$  is the angular displacement from the central image of the particular minimum or maximum under observation, and  $Z$  is constant characteristic of the particular maximum or minimum under observation. The reference by Millar<sup>22</sup> gives a table of the  $Z/\pi$  values for different orders of the spectrum.

## PROBLEMS AND QUESTIONS

1. Explain, in terms of the partial molar entropy of mixing, why the osmotic pressure of solutions of high-molecular-weight substances is greater than that calculated on the basis of ideal solution laws.

2. In 100 grams of 0.5  $M$  NaCl solution 1.427 grams of a protein were dissolved at the isoelectric point of the protein ( $pH$  5.2). The density of the NaCl solution was 1.024 at 25° C. This solution yielded an osmotic pressure of 10.65 cm. of water at 25° C. Assume ideal behavior, and calculate the molecular weight of the protein. Ans: 55,000

3. The 1.427 grams of the protein used in Problem 2 were adjusted to  $pH$  6.5 with dilute sodium hydroxide. NaCl and water were added to give 100 cc. of a solution which was 0.01  $M$  in respect to NaCl. The osmotic pressure of this solution at 25° C. was measured and found to be 14.65 cm. water at 25° C. Calculate the approximate charge on the protein molecules. Ans: 5.0

4. What kind of light-scatter measurements are necessary to decide whether the protein molecules are spheres, rods, or random coils?

<sup>22</sup> W. G. Millar, *Proc. Roy. Soc. (London)* B99, 264 (1926).

<sup>23</sup> E. Poulder, *Quart. J. Exptl. Physics* 23, 287 (1955).

<sup>24</sup> B. Lucke, M. G. Larrabee, and H. K. Hartline, *J. Gen. Physics* 19, 1 (1953).

## VISCOSITY and the FLOW of LIQUIDS

The study of the viscosity of colloidal solutions has led to much of value. It is important to know what information viscosity can give us and what it cannot. The methods available for its measurement are remarkably simple; almost too simple, because they are apt to be abused.

Viscosity is the resistance experienced by one portion of a liquid moving over another portion of the same liquid. The unit of viscosity, the poise, is defined as that viscosity such that unit force is required to cause two parallel liquid surfaces of unit area and unit distance apart to slide past one another with unit velocity, i.e.,

$$\text{Force} = \frac{\eta A u}{d} \quad (1)$$

where  $A$  is the area of the liquid surfaces at a distance  $d$  apart,  $u$  is the velocity difference in centimeters per second between the two liquid planes, and  $\eta$  is the coefficient of viscosity. The dimensions of viscosity are, therefore,  $Mt^{-1}L^{-1}$ .

Viscosity may also be defined in terms of the energy required to maintain a constant velocity gradient. The flow of a liquid is characterized by the fact that its maintenance requires the expenditure of energy at a rate that is proportional to the volume of the liquid and to the square of the velocity gradient. The constant of proportionality varies from one liquid to another and is called the coefficient of viscosity. This coefficient is identical, both numerically and dimensionally, with the coefficient of viscosity defined above in equation 1.

Occasionally and for special purposes another kind of viscosity is used. This is known as the kinematic viscosity and is equal to  $\eta/\rho$ , where  $\rho$  is the density of the liquid. The dimensions of kinematic viscosity are  $t^{-1}L^2$ .

### THEORY OF FLOW OF PURE LIQUIDS

Eyring<sup>1</sup> has proposed an ingenious molecular theory of the flow of liquids. This theory regards viscous flow as related to vaporization.

<sup>1</sup> H. Eyring, *J. Chem. Phys.* 4, 283 (1936); *Advances in Colloid Science*, Interscience Publishers, New York, 1949.

order for a liquid to flow, holes must be available for the molecules to move into. The production of such holes is akin to vaporization; accordingly, there should exist a relation between the energy of activation of the viscosity of a liquid and the heat of vaporization of the liquid. The flow of a liquid is regarded as a rate process, and from its dependence on temperature the energy of activation is calculated in the usual manner (Chapter 3). Experimental results show that such a relation does indeed exist.

With liquids containing molecules capable of hydrogen-bond formation, the energy of activation of viscosity decreases markedly with increasing temperature. The viscosity activation energy of these substances consists not only of the energy required to break what may be termed "physical bonds," but also of the energy required to break the hydrogen bonds. As the temperature is increased, the number of hydrogen bonds in a liquid diminishes and, accordingly, the energy of activation of viscosity also decreases. It is the presence of a network of hydrogen bonds that accounts for the very high viscosities of such compounds as glycerol and water.

In Table 1 are shown the coefficients of viscosity of several common liquids.

TABLE 1

## VISCOSITIES OF SOME COMMON LIQUIDS IN POISES

Liquid	Viscosity	Temperature in °C.
Benzene	0.00763	10
	0.00654	20
	0.00567	30
<i>n</i> -Butanol	0.02948	20
	0.01782	40
Ethanol	0.01752	10
	0.01716	20
	0.01681	30
Glycerol	25.18	8.1
	13.87	14.3
	8.30	20.3
	4.94	26.5
Heptane	0.00416	20
	0.00341	40
Methanol	0.00596	20
	0.00456	40
Water	0.015188	5
	0.013077	10
	0.011404	15
	0.010050	20
	0.010000	20 20
	0.008937	25
	0.008007	30
	0.007225	35
	0.006560	40



## FLOW OF LIQUIDS THROUGH CAPILLARIES

The fundamental law governing the flow of liquids through capillaries is that of Poiseuille, which, in the integrated form, is expressed as

$$V = \frac{\pi r^4 P}{8\eta l} \quad (2)$$

where  $V$  is the volume of liquid flowing in cubic centimeters per second,  $r$  is the radius of the capillary in centimeters,  $P$  is the difference in pressure between the two ends of the capillary and is expressed in dynes per square centimeter,  $\eta$  is the coefficient of viscosity in poises, and  $l$  is the length of the capillary in centimeters. Theory demands that the flow be streamlined or laminar and that there be no turbulence or eddies in the liquid as it flows. There is a critical pressure in every case above which turbulence appears. The critical pressure depends on the geometry of the system as well as on the density and viscosity of the liquid. The ratio of the inertial to the frictional force is  $(\rho/\eta)ua$ , where  $u$  is the fluid velocity and  $a$  is a characteristic linear dimension of the system. For capillaries,  $a$  is taken as the radius of the capillary. The expression  $(\rho/\eta)ua$  is called the Reynolds number<sup>2</sup> and is denoted by  $R$ . It is dimensionless and is independent of the units used. For a capillary and with the use of equation 2 the Reynolds number is

$$R = \frac{\rho r^3 P}{8\eta^2 l} \quad (3)$$

If  $R$  for a capillary is equal to or exceeds about 1000, turbulent flow will appear and a departure from Poiseuille's law will be noted. For other systems the Reynolds numbers are different.

## METHODS

The determination of the rate of flow through a capillary is the basis for an important method for the measurement of viscosity. In this method the time of flow of a known volume of liquid through a capillary under the influence of gravity is determined. Such an apparatus is diagrammed in Fig. 1.

Since the Ostwald viscometer is the most frequently used type of apparatus for the measurement of viscosity, it will be discussed in some detail. A definite volume of liquid is placed in the viscometer, and the level of the liquid is drawn above the top mark of the bulb by suction (see Fig. 1). The liquid is allowed to flow out freely, and the time ( $t$ ) required for the liquid

<sup>2</sup> O. Reynolds, *Trans. Roy. Soc. (London)* A174, 935 (1883).

level to drop from the upper mark to the lower mark is measured. The relation between viscosity and time of outflow is

$$\eta = C\rho t - f\left(\frac{\rho V}{lt}\right) \quad (4)$$

where  $C$  is a constant which involves the length and radius of the capillary. The second term on the right-hand side of the equation  $f(\rho V/lt)$  indicates that some function of  $\rho V/lt$  must be subtracted from  $C\rho t$  in order to obtain the viscosity. This term, the so-called kinetic correction, takes into account the motion of the liquid after it leaves the capillary. If  $t$  and  $l$  are sufficiently large, it can be neglected. The viscometer is calibrated with water or some other appropriate liquid whose viscosity is known exactly. If we neglect the kinetic correction, we have the relative viscosity

$$\eta_r = \frac{\eta}{\eta_0} = \frac{\rho t}{\rho_0 t_0} \quad (5)$$

where  $\eta$  is the coefficient of viscosity of the liquid whose viscosity we wish to measure,  $\eta_0$  is the coefficient of viscosity of the standard liquid,  $\rho_0$  is the density of the standard liquid, and  $t_0$  is the time of outflow of the standard liquid.  $\rho$  and  $t$  are the corresponding quantities of the liquid whose viscosity is being measured.

The most reliable method to determine whether the kinetic correction  $f(\rho V/lt)$  can be safely neglected is to measure the viscosity of two liquids whose viscosity is known. If the two measured viscosities bear the correct ratio to each other, and if the unknown viscosity has approximately the same value as that of the two known solutions, the kinetic correction is unnecessary. If not, it may be necessary to apply a kinetic correction although the error may be due to other causes. Indeed, if a kinetic correction is necessary, it is better to redesign the viscometer so that no such correction is required.

It is convenient to be able to calculate beforehand the dimensions of the capillary that will be acceptable for a given series of measurements. Directions for doing this have been given by Grüneisen<sup>3</sup> and have been clearly reviewed by Bungenberg de Jong.<sup>4</sup>

<sup>3</sup> E. Grüneisen, *Wiss. Abhandl. physik.-tech. Reichsanstalt* 4, 153 (1904).

<sup>4</sup> H. G. Bungenberg de Jong, *First Report on Viscosity and Plasticity*, Nordemann Publishing Co., Inc., New York, 1939.

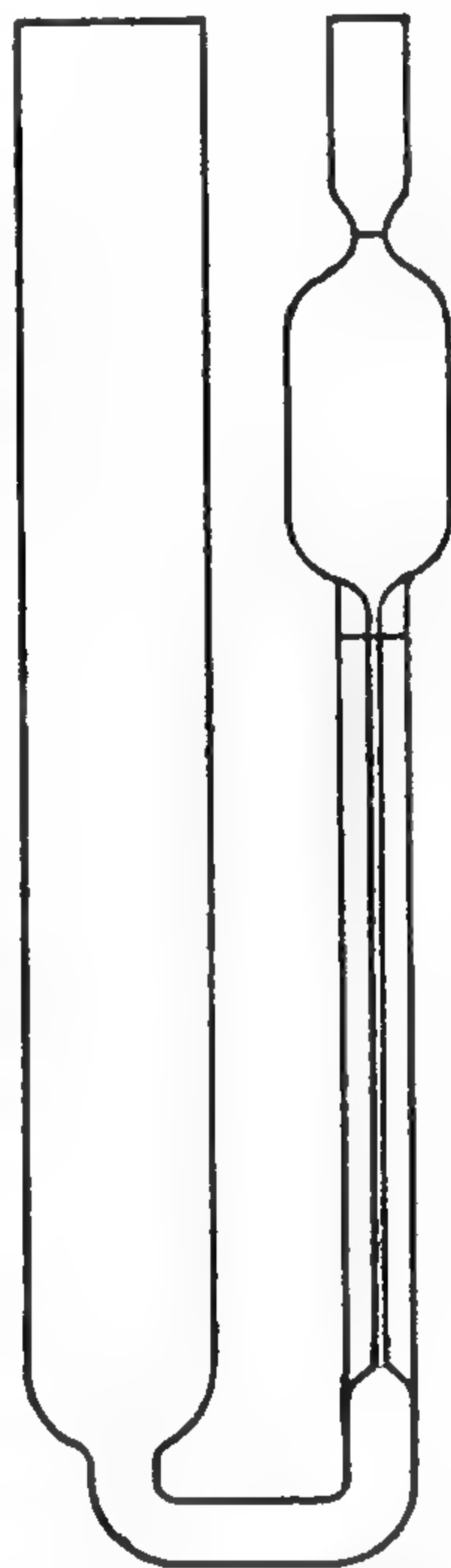


FIG. 1. Ostwald viscometer.

The mean linear velocity of flow of a liquid in a capillary is evidently

$$u = \frac{V}{\pi r^2} \quad (6)$$

Substituting the value of  $V$  from equation 6 into the Poiseuille equation 1, and remembering that  $h g \rho$  equals  $P$ , where  $h$  is the difference in liquid level in a viscometer and  $g$  is the acceleration of gravity, we have

$$u = \frac{h g \rho r^2}{8 \eta l} \quad (7)$$

Grüneisen found that the linear velocity must not exceed certain critical values if the departure from Poiseuille's law is not to be greater than a certain per cent. Grüneisen based his calculations on water at a temperature of 10° C. The author has recalculated his results to apply to water at 25° C. Table 2 gives the critical velocities of water at 25° C. for the indicated capillary dimensions. In order to calculate the critical velocities

TABLE 2

CRITICAL VELOCITIES OF WATER AT 25° C. AND THE PERCENTAGE FLOW ERROR ASSOCIATED WITH THESE CRITICAL VELOCITIES

	Diameter, in millimeters	Critical Velocity for 0.1% error, in centimeters per second	Critical Velocity for 1.0% error, in centimeters per second	Critical Velocity for 5.0% error, in centimeters per second
$l = 10 \text{ cm.}$	0.40	8.9	44.5	124
	0.45	6.5	34.3	92
	0.50	5.0	27.4	75
	0.55	3.8	22.2	61
	0.60	3.0	17.3	50
$l = 18 \text{ cm.}$	0.40	29.8	113	226
	0.45	21.6	88	179
	0.50	16.5	67	147
	0.55	12.8	53.5	122
	0.60	10.0	43.5	104

from the data in Table 2 for any other temperature and any other liquid, the velocities in Table 2 must be multiplied by  $\rho'/\eta'$  where  $\rho'$  is the density relative to water at 25° C. and  $\eta'$  is the viscosity relative to water at 25° C.

To illustrate the use to which the results in Table 2 can be put, consider water at 25° C. and a capillary 10 cm. long and 0.50 mm. in diameter. Substituting the appropriate values in equation 7, we find

$$u = 3.4h \quad (8)$$



At  $h$  equal to 10 cm., the linear velocity will be 34.4 cm. per second. Referring to Table 2, we find that such a capillary has a critical velocity of 27.4 cm. per second for a flow error not exceeding 1 per cent. Accordingly, we conclude that in our case the flow error will be slightly in excess of 1 per cent. One has a certain margin of error in applying Table 2. As the value of  $h$  constantly diminishes as the liquid flows through the viscometer, the flow error will be somewhat smaller than that calculated. Also, the Ostwald viscometer is used to compare the flow of a standard liquid with that of another liquid, and the flow errors from the two liquids tend to cancel each other. In designing a viscometer, however, it is better to forget about the margin of error and design the instrument so that its calculated flow error does not exceed the other errors involved in the viscosity determination. Actually, kinetic flow errors begin to be appreciable at much lower pressures than the pressures at which turbulent flow begins (equation 3).

In addition to the kinetic correction mentioned above, the Ostwald viscometer is subject to three other errors: (1) drainage error, (2) working-volume correction, and (3) surface-tension effects. The ideal design of a viscometer strikes a compromise with all these disturbing factors.

Jones and Stauffer<sup>5</sup> have shown that the drainage error is negligible except for very viscous liquids or for viscometers with very short times of outflow.

The surface-tension correction may be important for liquids whose surface tension differs greatly from that of the calibrating liquid.<sup>6</sup> Bull<sup>7</sup> found that solutions of egg albumin showed some very anomalous flow relations which he attributed to surface films of this protein. The working-volume correction refers to error inherent in placing a precise volume of liquid in the viscometer. Ordinarily the error involved here is much smaller than the other types of errors.

Anyone who plans to use the Ostwald viscometer for accurate work should consult the excellent series of papers by Jones and co-workers in the *Journal of the American Chemical Society*. The paper by Bungenberg de Jong<sup>4</sup> to which reference has already been made is also helpful. In conclusion of this discussion, it is well to emphasize that two absolute necessities for satisfactory viscosity measurements are cleanliness of the viscometer and an accurate temperature control.

The Couette viscometer employs a rotating cylinder containing the liquid. A smaller cylinder is suspended in the liquid by means of a fine wire. The torque produced on the suspended cylinder by the rotation of the outer cylinder is recorded by a beam of light thrown on a mirror at-

<sup>5</sup> G. Jones and R. E. Stauffer, *J. Am. Chem. Soc.* 59, 1630 (1937).

<sup>6</sup> G. Jones and H. J. Fornwalt, *J. Am. Chem. Soc.* 60, 1683 (1938).

<sup>7</sup> H. B. Bull, *J. Biol. Chem.* 133, 39 (1940).

tached to the suspension wire. The light is reflected from the mirror on a graduated scale. The type of flow obtained in a Couette viscometer is simpler than that in an Ostwald viscometer, and the theoretical treatment is less involved. Also, very much smaller flow gradients can be obtained conveniently. Unfortunately, the temperature control in this type of apparatus presents a real difficulty; also, the apparatus has to be very exactly made, the services of an expert machinist being required. Björnstahl and Snellman<sup>8</sup> report the construction of a Couette viscometer. There are several commercial adaptations of the Couette principle. Great accuracy cannot, however, be expected from them.

The Hess viscometer<sup>9</sup> requires very small volumes of fluid and has found some favor in the measurement of the viscosity of blood. It consists of two horizontal capillaries. One of the capillaries is partly filled with water and the other partly filled with blood. The two capillaries are connected to a rubber bulb which can be squeezed. The distance traveled along the capillary by the water is compared with that traveled by the blood. The ratio of the two distances traveled is equal to the inverse ratio of the two viscosities. The Hess viscometer is commercially available. Its accuracy is not of a high order.

#### VISCOSITY OF COLLOIDAL SOLUTIONS

It is evident that, if spherical colloidal particles are suspended in a liquid, the viscosity of the liquid will increase, owing to the disruption of the uniform flow gradients of the liquid. On this basis Einstein<sup>10</sup> formulated his well-known equation for the viscosity of a suspension of spherical particles. The equation is

$$\eta = \eta_0 \left[ \frac{1 + 0.5\phi}{(1 - \phi)^2} \right] \quad (9)$$

where  $\eta$  is the coefficient of viscosity of the suspension and  $\eta_0$  is that of the dispersion medium.  $\phi$  is the relative volume concentration of the suspension; it is equal to the total volume of the suspension divided into the total volume of the suspended material.

Guth<sup>11</sup> has outlined the assumptions upon which the Einstein equation is based. They are as follows: (1) The suspended spheres are large compared with the molecules of the dispersion medium but small compared with the dimensions of the viscometer. (2) The dispersion medium is incompressible. (3) The suspended particles are rigid and are completely

<sup>8</sup> Y. Björnstahl and O. Snellman, *Kolloid-Z.* 86, 223 (1939).

<sup>9</sup> W. R. Hess, *Kolloid-Z.* 27, 154 (1920).

<sup>10</sup> A. Einstein, *Ann. Physik* 19, 289 (1906); 34, 591 (1911).

<sup>11</sup> E. Guth, *Kolloid-Z.* 74, 147 (1936).



wet by the dispersion medium. (4) Gravitational forces acting on the particles can be neglected. (5) The velocity of flow of the medium is so small that the Reynolds number of the particle is much less than unity; that is

$$R_p = \frac{\rho}{\eta_0} a u \ll 1 \quad (10)$$

where  $a$  is the radius of the particle,  $u$  is the linear velocity of streaming of the medium,  $\rho$  is the density of the particle, and  $\eta_0$  is the viscosity of the medium. (6) The concentration of the suspension is so small that there is no interference of one particle with another. (7) The suspended particles are distributed at random throughout the medium. (8) It is assumed that the flow, with the exception of that in the immediate neighborhood of the particles, is static and does not change significantly except within a volume that is large compared with the radius of the particle.

The Einstein equation can be expanded into a power series which is

$$\frac{\eta}{\eta_0} = 1 + 2.5\phi + 4\phi^2 + 5.5\phi^3 + \dots \quad (11)$$

Owing to particle interaction higher terms than the first are without significance and

$$\frac{\eta}{\eta_0} = 1 + 2.5\phi \quad (12)$$

which is the form in which one frequently sees the equation written. Eirich, Bunzl, and Margaretha<sup>12</sup> have given adequate experimental demonstration that the Einstein equation is valid for very dilute suspensions of spherical particles, thus showing clearly that the first coefficient of the series is 2.5.

Vand<sup>13</sup> has considered the influence of the proximity of the wall of the viscometer, and the interaction of the particles, and finds that

$$\eta_r = 1 + 2.5\phi + 7.35\phi^2 + \dots \quad (13)$$

This equation was tested by means of suspension of small glass spheres suspended in mixtures of a saturated solution of zinc iodide, water, and glycerol.<sup>14</sup> He found that he could express his experimental results up to a volume concentration of about 50 per cent by the series

$$\eta_r = 1 + 2.5\phi + 7.17\phi^2 + 16.2\phi^3 + \dots \quad (14)$$

<sup>12</sup> F. Eirich, M. Bunzl, and H. Margaretha, *Kolloid-Z.* 74, 276 (1936).

<sup>13</sup> V. Vand, *J. Phys. & Colloid Chem.* 52, 277 (1948).

<sup>14</sup> V. Vand, *J. Phys. & Colloid Chem.* 52, 300 (1948).



The term  $\eta/\eta_0 - 1$  or  $\eta_r - 1$  is called the specific viscosity and is expressed by the symbol  $\eta_s$ . Dividing equation 14 by  $\phi$ , we have

$$\frac{\eta_s}{\phi} = 2.5 + 7.17\phi + 16.2\phi^2 + \dots \quad (15)$$

Equation 14 is in excellent agreement with his theoretical equation (equation 13). It would appear from this agreement and additional evidence to be mentioned presently that Vand's equation is in all probability very nearly, if not actually, the correct expression for the viscosity of a suspension of rigid, spherical particles.

It is clear that, if the suspended particles are hydrated, Vand's equation offers a direct means for calculating the hydration. If we plot  $\eta_s/\phi$  against  $\phi$ , we should obtain a straight line, at least for the lower concentrations. From the intercept on the  $\eta_s/\phi$  axis we obtain  $\eta_s/\phi$  at infinite dilution. If the particles are spherical,  $\eta_s/\phi$  should equal 2.5. We, therefore, calculate the additional volume, i.e., hydration, which must be added to the volume of the suspended particles in their dry condition to yield 2.5 for the intercept. The difficulty is, of course, that the majority of suspensions do not have spherical particles and the application of Vand's equation to non-spherical particles is meaningless.

Kraemer<sup>15</sup> introduced the term intrinsic viscosity  $[\eta]$  which is equal to  $\eta_s/c$  as  $c$  approaches infinite dilution, and he suggests that the readiest way of calculating the intrinsic viscosity from experimental data is by the relation

$$[\eta]_{c \rightarrow 0} = \frac{\ln \eta_r}{c} \quad (16)$$

where  $c$  is expressed in grams of solute per 1.00 cc. of solution. Actually, it is sometimes easier to evaluate  $[\eta]$  by the relation

$$\left( \frac{\eta_s}{c} \right)_{c \rightarrow 0} = [\eta] \quad (17)$$

the value of  $[\eta]$  being obtained from the intercept of the curve on the  $\eta_s/c$  axis when  $\eta_s/c$  is plotted against  $c$ . The intrinsic viscosity in equation 17 is the weight intrinsic viscosity. The corresponding volume intrinsic viscosity would be  $\left( \frac{\eta_s}{\phi} \right)_{\phi \rightarrow 0}$  and for unhydrated rigid spheres is equal to 2.5.

#### PARTICLE ASYMMETRY AND VISCOSITY

It was realized rather early that there must be a relation between the asymmetry of the suspended particles and the viscosity of a suspension.

<sup>15</sup> E. O. Kraemer, *J. Ind. Eng. Chem.* 30, 1200 (1938).

The particles are turning and twisting in Brownian motion, and they thus appear to occupy a larger volume than they actually do. The twisting of the particle interrupts the streamlines of flow and requires additional expenditure of work to maintain a given velocity of flow. This work appears as an increase in the viscosity.

Staudinger<sup>16</sup> approached the problem of asymmetry from an experimental point of view. Using a series of long-chain hydrocarbons, alcohols, and acids dissolved in such inert solvents as benzene or carbon tetrachloride, he found the specific viscosity of the solutions to be a function of the number of groups of "submolecules" contained in the chain. For a paraffin hydrocarbon, the submolecule is the CH<sub>2</sub> group; for cellulose, it is the glucose anhydride residue. He proposes the equation

$$\eta_s = K n C \quad (18)$$

where  $K$  is a constant for a given series of compounds,  $n$  is the number of submolecules per molecule, and  $C$  is the concentration of the solution in submolecules per liter. For example, for a hydrocarbon,  $C$  would be equal to the concentration in grams per liter divided by 14, the molecular weight of the submolecule. The Staudinger relation has been extensively investigated by numerous other workers and has proved of great value in many technical and industrial problems. This approach, however valuable it may be to industry, still does not contribute greatly to the understanding or even description of the general problem of the relation between asymmetry of colloidal particles and viscosity.

The theoretical relation between particle asymmetry and viscosity is quite complicated and has been approached by a number of workers. Simha<sup>17</sup> has apparently succeeded in expressing this relation for rigid prolate and for rigid oblate ellipsoids of revolution more completely than anyone else. Figure 2 shows a graph of the ratio of major to minor axis for ellipsoids of revolution against the volume intrinsic viscosity.

The problem of the viscosity of long, flexible chain molecules has been treated by Huggins,<sup>18</sup> Debye,<sup>19</sup> and Kirkwood and Riseman.<sup>20</sup> Kuhn and Kuhn<sup>21</sup> have considered the deformation produced by flow gradients acting on long flexible molecules forming random coils. Most high biopolymers contain so many centers capable of hydrogen-bond formation that the interaction of portions of these molecules with portions of the same molecule

<sup>16</sup> H. Staudinger, *Ber.* 68, 2320 (1935).

<sup>17</sup> R. Simha, *J. Phys. Chem.* 44, 25 (1940).

<sup>18</sup> M. L. Huggins, *J. Phys. Chem.* 42, 911 (1938); 43, 4 (1939).

<sup>19</sup> P. Debye, *J. Chem. Phys.* 16, 570 (1948).

<sup>20</sup> J. G. Kirkwood and J. Riseman, *J. Chem. Phys.* 16, 565 (1948).

<sup>21</sup> W. Kuhn and H. Kuhn, *J. Colloid Sci.* 3, 11 (1948).

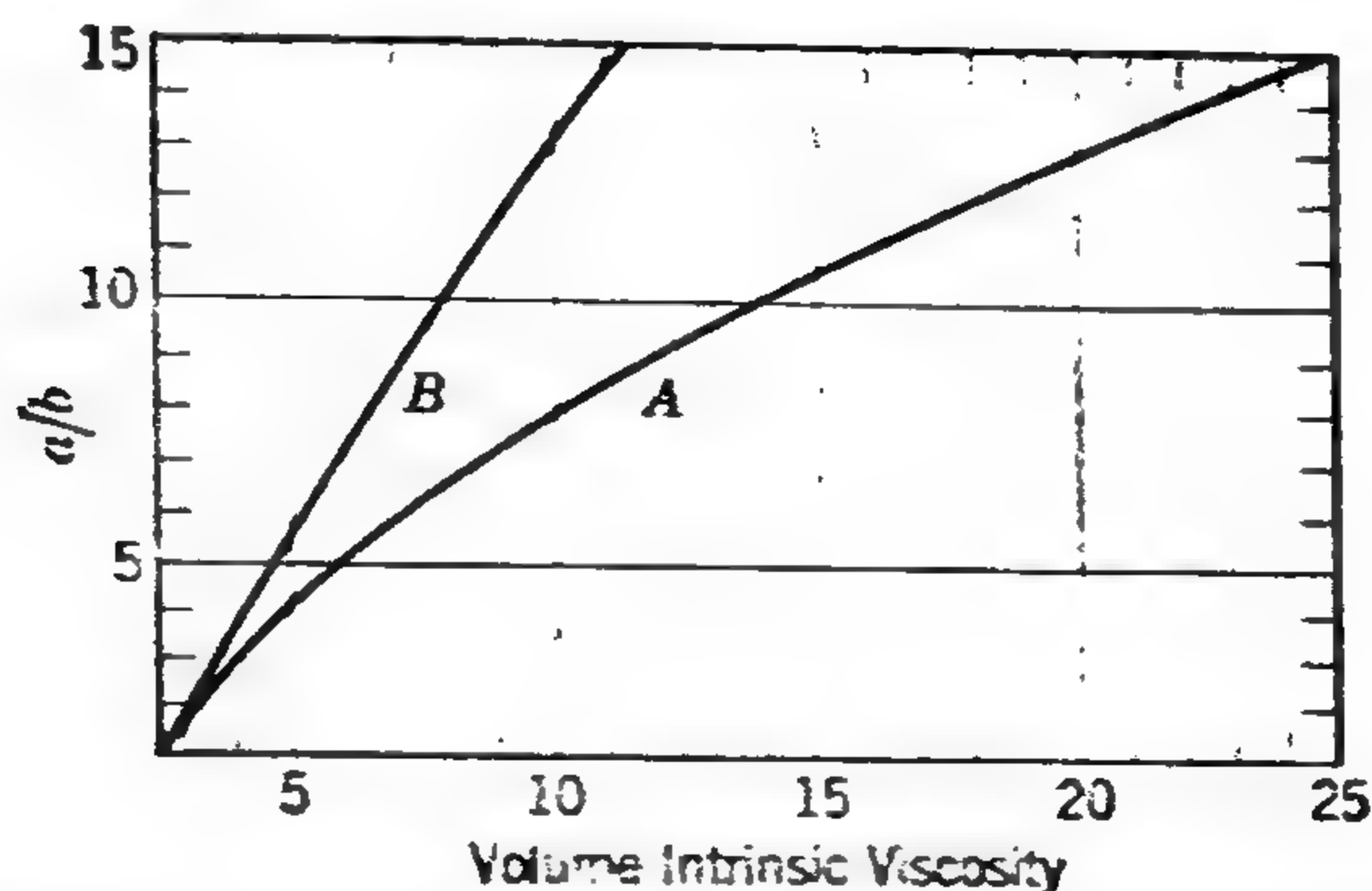


FIG. 2. Plot of Simha's viscosity equation showing relation between particle asymmetry and volume intrinsic viscosity. (A) Prolate ellipsoids of revolution. (B) Oblate ellipsoids of revolution.

probably gives rise to rather rigid, stiff molecules in solution. It would appear that Simha's approach to this problem for this class of molecules is the more realistic.

#### VISCOSITIES OF PROTEIN SOLUTIONS

The question of asymmetry of particles is particularly vital to the protein chemist. There is no way at the present time of obtaining unambiguous values for the asymmetries of protein molecules. From the above discussion, it is clear that a relation should exist between the asymmetries of protein molecules and the viscosities of their solutions. This realization has prompted considerable work on the viscosity of dilute protein solutions. The problem is not a simple one. In addition to the innate difficulty of finding a relation between particle asymmetry and viscosity there are two other difficulties. No one knows what form the asymmetries of protein molecules assume: i.e., are they cylinders, disks, prisms, prolate ellipsoids, oblate ellipsoids, etc.? Also unknown is the influence of hydration. In order to make any sense out of viscosity measurements, we must know the volume occupied by the dispersed phase. It is an easy matter to measure the volume occupied by a given weight of dry protein, but, when dissolved in water, proteins take up an unknown amount of water and, accordingly, occupy a larger volume dissolved than in their dry state. The manner in which hydration increases the effective volume is also unknown. For example, is the swelling uniform over the whole molecule, or does it center about the major or about the minor axis of asymmetry? We have very inconclusive answers to these questions. After we have discussed diffusion, we shall return to these matters again.

Treñers<sup>2</sup> reported that the viscosity of a protein solution could best be

<sup>2</sup> H. P. Treñers, *J. Am. Chem. Soc.* 62, 1405 (1940).



expressed in terms of fluidity. Fluidity is the reciprocal of viscosity. The relation between fluidity and concentration, according to Treffers, is

$$f_r = \frac{f}{f_0} = (1 - KC) \quad (19)$$

where  $f_r$  is the relative fluidity and is equal to  $1/\eta_r$ .  $f$  is evidently equal to  $1/\eta$ , and  $f_0$  is equal to  $1/\eta_0$ .  $C$  is the concentration of the protein in grams per 100 cc. of solution. Treffers expressed concentration in grams per 100 cc. solvent. From a prior reasoning one would anticipate that a better relation would be obtained by expressing concentration in terms of the volume of the solution and not in terms of the volume of the solvent. This is, indeed, found to be true.  $K$  is a constant which is dependent on the nature of the protein. Bingham and Roepke<sup>23</sup> have found this relation to hold for solutions of fibrinogen. Equation 19 is evidently equivalent to the expression

$$\eta_r = \frac{1}{(1 - KC)} \quad (20)$$

If we expand equation 20 into a power series, we have

$$\eta_r = 1 + KC + K^2C^2 + K^3C^3 + \dots \quad (21)$$

This is a rather important and interesting conclusion. If we know the first coefficient of the series, we can immediately write down the coefficients for any other terms. Since  $\phi$  is proportional to  $C$  and since for a spherical particle the volume intrinsic viscosity is equal to 2.5, we have the following series for a suspension of spherical particles:

$$\eta_r = 1 + 2.5\phi + 6.25\phi^2 + 15.6\phi^3 + \dots \quad (22)$$

The empirical equation 22 compares very favorably with the theoretical equation of Vand. This approach emphasizes the significance of the first coefficient in describing the viscosity of any suspension; in this coefficient is included the influence of both hydration and asymmetry. The value of  $K$  in equation 21 is evidently equal to the intrinsic viscosity. In Chapter 14 will be found a table showing the intrinsic viscosities of some proteins.

## ELECTROVISCOUS EFFECT

Falkenhagen and Dole<sup>24</sup> found that the viscosity of dilute electrolyte solutions could be expressed by the relation

$$\eta = \eta_0(1 + A\sqrt{C}) \quad (23)$$

where  $C$  is the equivalent concentration of the electrolyte and  $A$  is a con-

<sup>23</sup> E. C. Bingham and R. R. Roepke, *J. Am. Chem. Soc.* **64**, 1204 (1942).

<sup>24</sup> H. Falkenhagen and M. Dole, *Physik. Z.* **30**, 611 (1929).

stant involving the viscosity of the liquid, the dielectric constant, the temperature, and the valence and the mobility of the ions. This equation was derived on the assumption that the increase in viscosity arises from the work required to disrupt the ionic atmosphere around the ions and to move the positive and negative ions relative to each other.

The electroviscous effect due to charged colloidal particles was first treated by Smoluchowski,<sup>25</sup> who, shortly before his death, published an equation without derivation which was supposed to describe the electroviscous effect. Later Krasny-Ergen<sup>26</sup> reconsidered the question and derived an equation that is substantially the same as Smoluchowski's differing from it only in the magnitude of a numerical coefficient. The Krasny-Ergen equation is

$$\eta_r = 1 + 2.56 \left[ 1 + \frac{3}{2} \frac{1}{\kappa \eta_0 a^2} \left( \frac{D(\Gamma_i - \Gamma_a)}{2\pi} \right)^2 \right] \quad (24)$$

where  $\kappa$  is the specific conductance of the dispersion medium,  $\eta_0$  is its viscosity,  $D$  is the dielectric constant of the medium, and  $a$  is the radius of the spherical particle. According to Krasny-Ergen,  $\Gamma_i$  is the potential difference between the surface of the particle and some point in the liquid where the charge density is zero, and  $\Gamma_a$  is the potential difference between the surface of the particle and the interior of the particle. Thus  $\Gamma_i - \Gamma_a$  is the total difference in potential between the interior of the particle and the interior of the liquid. If this is true,  $\Gamma_i - \Gamma_a$  cannot be replaced by  $\zeta$ , which is the potential across the diffuse double layer only. This appears to be an error of definition, however, and it is the custom to assume that  $\Gamma_i - \Gamma_a$  is the potential across the double layer and thus equal to the  $\zeta$ -potential. The Krasny-Ergen equation involves all the assumptions of the Einstein equation in addition to the following: (1) the charge on the small sphere is uniformly distributed over the surface of the spheres; (2) the particles are non-conducting; (3) the thickness of the double layer is thin in comparison with the radius of the particle; (4) the streaming potential set up by the motion of the particles in respect to the liquid must exert no back pressure on the liquid; no electroosmotic effects.

For the purposes of testing equation 24 Briggs<sup>27</sup> writes it in the following form

$$\frac{1}{\eta_r} = \frac{1}{K\phi} - \frac{3D^2}{8\pi^2 r^2} \frac{\zeta^2}{\lambda(\eta_{sol} - \eta_0)} \quad (25)$$

when  $1/\eta_r$  is plotted against  $\zeta^2/\lambda(\eta_{sol} - \eta_0)$ , the intercept of the straight line on the ordinate is equal to  $1/K\phi$ , and the slope of the line is equal to

<sup>25</sup> M. v. Smoluchowski, *Kolloid-Z.* 18, 194 (1916).

<sup>26</sup> W. Krasny-Ergen, *Kolloid-Z.* 74, 172 (1936).

<sup>27</sup> D. R. Briggs, *J. Phys. Chem.* 45, 866 (1941).



$-3D^2/8\pi^2r^2$ . Briggs and co-workers studied the viscosities of solutions of gum arabic,<sup>27</sup> of casein,<sup>28</sup> and of  $\beta$ -lactoglobulin.<sup>29</sup> These workers obtained linear relations in all their plots, and to this extent equation 24 appears valid. However, the fact that the slopes of these lines varied widely for different concentrations of the same substance is not in agreement with equation 24.

Figure 3 shows the specific viscosity divided by the volume of egg albumin (calculated from the partial specific volume of this protein) plotted as a function of pH in the presence and absence of neutral salt.<sup>30</sup>

Since the approximate radius of egg albumin molecules is known and the electrophoretic mobility as well as the specific conductance of the solutions shown in Fig. 3 were measured, all the necessary information is available to test equation 24. The measured viscosities were from 0.7 to 10 per cent of those predicted by equation 24. It is true that this is not an unambiguous test of this equation, since the thickness of the electrical double layer is of the order of magnitude of the size of the albumin molecule; whether the equation is valid has not been settled, but it appears improbable that it is correct.

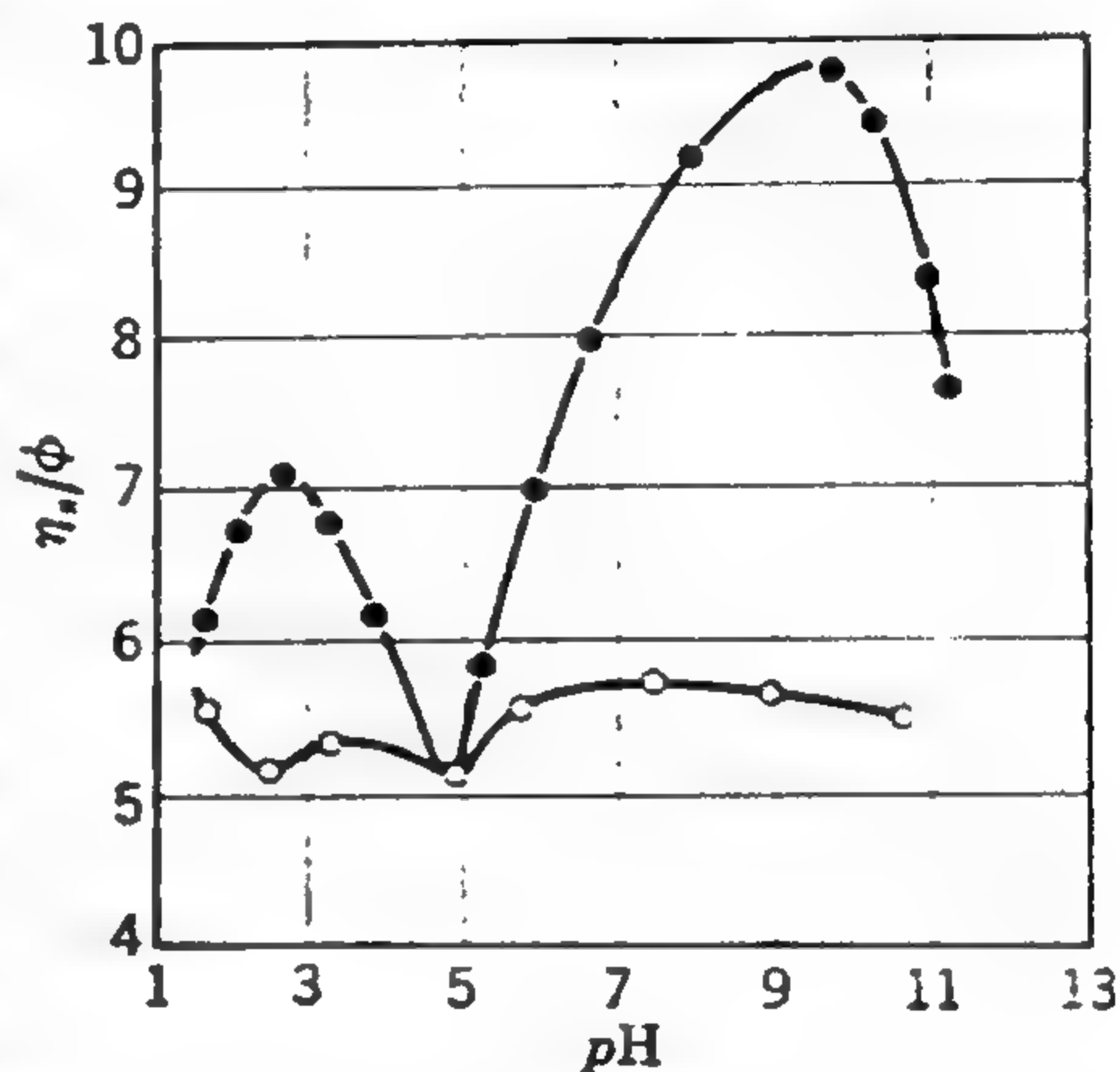


FIG. 3.  $(\eta_{sp}/c)$  as a function of pH of a 0.2 per cent egg albumin solution. Solid circles, in absence of NaCl. Open circles, in presence of 0.01 M NaCl on basic side of isoelectric point and 0.02 M NaCl on acid side.

Finkelstein and Cursion<sup>31</sup> have proposed a fairly complicated equation expressing the electroviscous effect and taking into consideration the distribution of ions around the suspended particle as well as the electro-osmotic effect due to the motion of the charged particle. Their theory has not been tested.

Markovitz and Kimball<sup>32</sup> discuss the influence of salts on the viscosity of solutions of polyacrylic alcohol and consider in addition to the strictly electrical effects the possibility that the polymer molecule unfolds, owing to the electrostatic charges and coils up in the presence of electrolytes.

The interest of the experimenter usually lies not in a study of the elec-

<sup>27</sup> C. L. Hankinson and D. R. Briggs, *J. Phys. Chem.* **45**, 943 (1941).

<sup>28</sup> D. R. Briggs and Martin Havig, *J. Phys. Chem.* **48**, 1 (1944).

<sup>29</sup> H. B. Bull, *Trans. Faraday Soc.* **36**, 80 (1940).

<sup>31</sup> B. N. Finkelstein and M. P. Cursion, *Acta Physicochim. U.R.S.S.* **17**, 1 (1942).

<sup>32</sup> H. Markovitz and G. E. Kimball, *J. Colloid Sci.* **5**, 115 (1950).



troviscous effect as such but only as a source of ambiguity; he wishes to know how to eliminate it. The most direct way of avoiding an electroviscous effect is to increase the concentration of electrolyte until a point is reached at which the measured specific viscosity is independent of salt concentration. In doing this the hydration of the colloid has been changed, but there is no other way out.

### VISCOSITY IN BIOLOGICAL SYSTEMS

Wherever there is a transfer of liquids in biological systems, thought must be given to the viscosity of those liquids. A classic example of such a transfer is the motion of blood in the arteries, veins, and capillaries of higher animals. Analysis shows that the velocity of flow of blood through the great aorta is so great at times that the flow approaches a condition of turbulence.

The red cells contribute greatly to the viscosity of blood. At 37° C. the viscosity of whole human blood varies between 3 and 4 centipoises; that of the plasma is about 1 centipoise. The viscosity of whole blood decreases with increasing rate of shear, the effect being due principally if not entirely to an orientation of the red cells in the flow gradient.

A streaming motion is frequently observed in protoplasm. A very elegant example of protoplasmic streaming is shown by the myxomycete, *Physarum polycephalum*, and Kamiya and Abe<sup>33</sup> have devised a simple and effective apparatus for studying not only the tendency to flow but also the electrical potential between the ends of the protoplasm. Two masses of protoplasm connected by an unbroken strand of protoplasm are placed in separate air-tight compartments. The difference in air pressure between the two compartments, which is just sufficient to prevent the protoplasmic streaming, is determined. Simultaneously, the potential difference between the two masses of protoplasm is measured with appropriate calomel half-cells. The counterpressure varies in a rhythmic manner with time, usually between 10 cm. to -10 cm. of water pressure. The potential difference varies between about 3 and -5 millivolts, and, furthermore, its rhythm follows the pressure rhythm by 14° on the average, the difference diminishing with increase in temperature. The length of the cycle varies but is on the average 3 to 4 minutes. There is indeed a remarkable correspondence between the variation of the magnitude of the potential difference and the pressure difference. It is concluded that the peculiar wave forms observed with counterpressure as well as the potential difference with time are due to interference of several rhythmic processes occurring simultaneously.

<sup>33</sup> N. Kamiya and S. Abe, *J. Colloid Sci.* 5, 149 (1950).

Loewy<sup>34</sup> has proposed a theory of protoplasmic streaming based on the idea that the streaming liquid contains protein molecules capable of contraction. The fiber protein molecules attach themselves to the protoplasmic surface external to the stream and then undergo contraction. The result would be that flow would be produced.

It is evident that the measurement of the viscosity of cell interiors presents difficulties. In the first place, the viscosity of a cell interior is not uniform throughout; furthermore, its value is greatly dependent on the physical and chemical changes. Any technique that is used to measure this viscosity is very likely to change the viscosity. One method is to observe the rate of motion of particles inside the cells under the influence of centrifugal force and to apply Stokes' formula

$$V = \frac{8\pi^2 n^2 r (\sigma - \rho) a^2}{9\eta} \quad (26)$$

where  $n$  is revolutions of the centrifuge per second,  $r$  is the radius of the circle through which the tube turns,  $\sigma$  is the specific gravity of the spherical particle,  $\rho$  is the specific gravity of the cell interior,  $a$  is the radius of the particle inside the cell, and  $V$  is the rate of movement of the particles in centimeters per second. Heilbrunn<sup>35</sup> describes the centrifuge method, as well as the Brownian movement method.

This last method depends on the measurement of the excursion made by a particle in the cell interior as a result of the heat motion of the particle and the Einstein equation applied

$$d = \frac{RTt}{3\pi\eta a.N} \quad (27)$$

where  $d$  is the distance traveled by the granule in any given plane in time  $t$ ,  $T$  is the absolute temperature,  $R$  is the gas constant,  $a$  is the radius of the particle, and  $N$  is Avogadro's number.

The general range of protoplasmic viscosity is 3 to 5 centipoises, although the viscosity in the neighborhood of the cell cortex can be very much greater. The stimulation of protoplasm by whatever agent tends to increase its viscosity very greatly.

<sup>34</sup> A. G. Loewy, *Proc. Am. Phil. Soc.* 93, 326 (1949).

<sup>35</sup> L. V. Heilbrunn, *Physical Research Methods*, edited by F. M. Uber, Interscience Publishers, Inc., New York, 1950.

## PROBLEMS AND QUESTIONS

1. Define the terms relative viscosity, specific viscosity, and volume intrinsic viscosity. How may each of these be determined experimentally?

2. Egg albumin was dissolved in a series of 0.03 *M* phosphate buffers at pH 7.0. The times of outflow in an Ostwald viscometer as well as the relative densities of the protein solutions were measured at 25° C. with the following results:

Grams Protein per 100 cc.	Time of Outflow, in seconds	Relative Density
0.00	96.17	1.0000
0.53	98.12	1.0014
1.62	102.26	1.0041
3.24	108.58	1.0080
4.85	115.57	1.0120
6.47	123.74	1.0158

Assume a hydrated density of 1.26 for egg albumin and calculate the volume intrinsic viscosity. Estimate the ratio of the major to the minor axis of the protein molecule, assuming a prolate ellipsoid of revolution and using Simha's equation.

*Ans.:*  $[\eta] = 5.13$ ;  $a/b = 4.2$ .

3. What is meant by turbulent flow and what are the conditions for the onset of turbulent flow?



## DIFFUSION

It can be said fairly that the diffusion of substances through cell membranes as they pass into and out of cell interiors is a fundamental biological problem. Quite apart, however, from the physiological aspects, diffusion measurements are capable of yielding valuable information about molecules. It is this phase of the problem that we wish to consider at this point.

Diffusion was the subject of early study. In 1855, Fick enumerated his first law of diffusion, and shortly afterwards Thomas Graham, the father of colloid chemistry, distinguished between "crystalloids" and "colloids" on the basis of diffusion. Colloidal substances were not supposed to diffuse, in contrast to crystalloidal substances. We now know that the difference in diffusibility is one of degree and not of kind. The large colloidal particles simply diffuse more slowly.

It is not our purpose to enter into an extended exposition of the theory of diffusion. This is treated in some detail in the review by Williams and Cady.<sup>1</sup> We should, however, have clearly in mind a physical picture of diffusion. Consider Fig. 1, which is taken from the excellent review of protein diffusion by Neurath.<sup>2</sup> The solution at concentration  $C_0$  is in contact with the pure solvent. Diffusion takes place only in a horizontal direction. Under these conditions

$$\text{Rate of diffusion} = -DA \frac{dC}{dx} \quad (1)$$

where  $A$  is the cross-sectional area of the diffusion column and  $dC/dx$  is the concentration gradient.  $D$  is the diffusion constant which is characteristic of the solute molecules in question.

If the slopes of the concentration-distance curves shown in the center section of Fig. 1 are plotted against the distance, a family of curves is obtained as is shown in the bottom section of Fig. 1. These curves have the shapes of Gaussian distribution curves and are identical with one another

<sup>1</sup> J. W. Williams and L. C. Cady, *Chem. Revs.* 14, 171 (1934).

<sup>2</sup> H. Neurath, *Chem. Revs.* 30, 357 (1942).

## DIFFUSION

respect to their areas. Curves of this type follow the equation

$$\frac{dC}{dx} = \frac{C}{2\sqrt{\pi Dt}} e^{-x^2/4Dt} \quad (2)$$

where  $t$  is the elapsed time after the forming of the boundary between the solvent and solution, and  $C$  is the concentration at any distance  $x$  from the

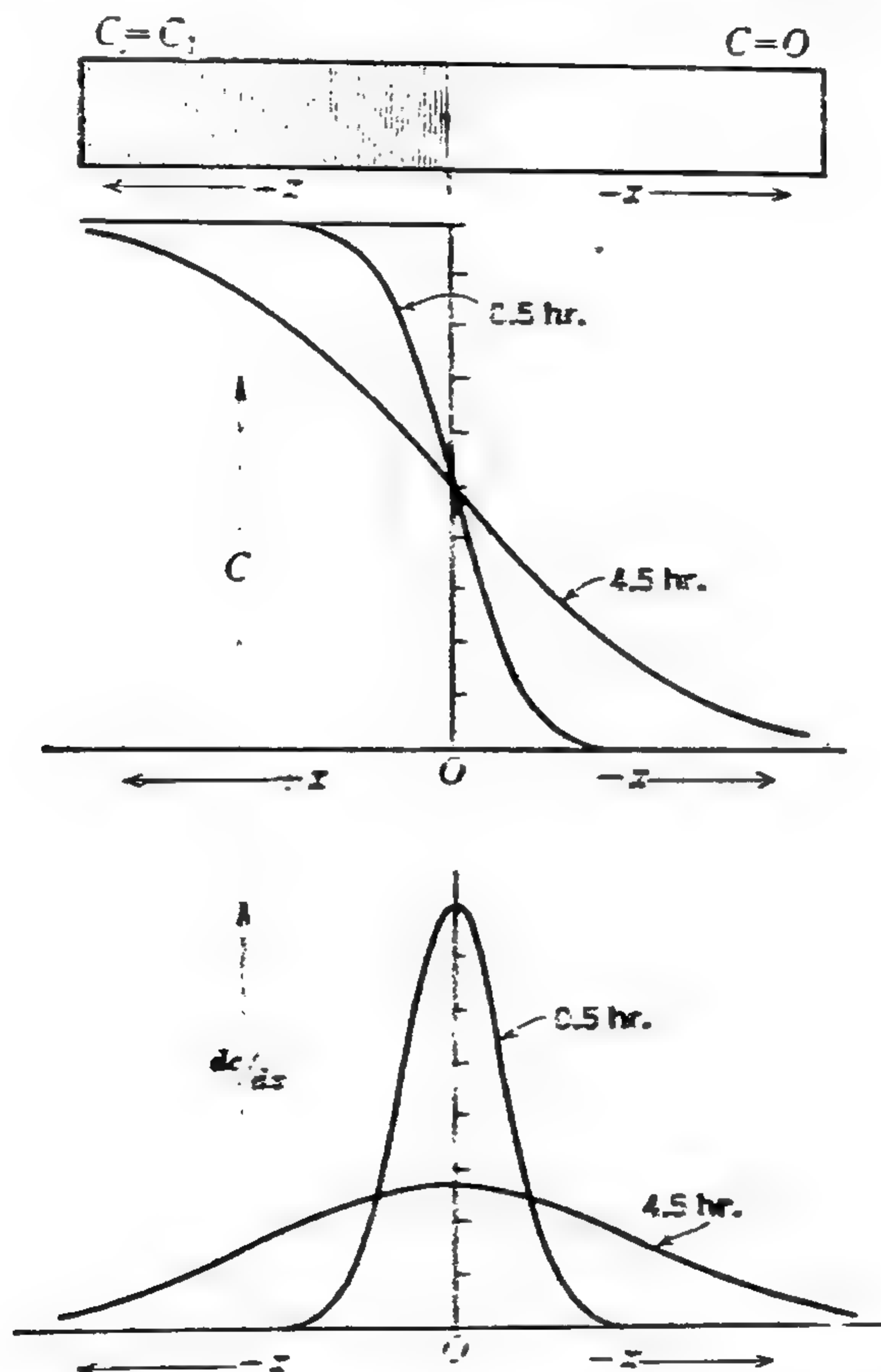


FIG. 1. Relation between concentration and distance of migration in diffusion column. Top section: graphical illustration of the diffusion column. Center section: relation between concentration and distance of migration. Bottom section: relation between concentration gradient and distance of migration. The curves as drawn refer to diffusion times of 0.5 and 4.5 hours, respectively. (Neurath.)

original boundary. The sign of  $x$  is negative in the direction of the solvent and positive towards the solution.  $e$  is the base of the natural logarithms. We shall describe presently how this equation can be used to evaluate the diffusion constant.

## MEASUREMENT OF DIFFUSION CONSTANTS

Of the various ways of measuring diffusion constants, we shall content ourselves with a discussion of only two. The first method is based on the chemical analysis of two solutions of different concentration of the solute separated by a porous disk; we shall call it the porous-disk method. The second method involves a measurement of the manner in which the index of refraction of light varies at a boundary between the solution and the solvent; we shall call it the refractometric method.

The porous-disk method was developed largely by Northrop and Anson<sup>3</sup> and by McBain and Liu.<sup>4,5</sup>

By definition, the diffusion constant is the quantity of material that diffuses per second across a surface 1 sq. cm. in area under a unit concentration gradient; hence

$$D = \frac{dQ}{A \, dt \, dC/dx} \quad (3)$$

where  $dQ$  is the quantity of diffusion material that passes across a plane of area  $A$  in time  $dt$  under a concentration gradient  $dC/dx$ . Substituting dimensional quantities in equation 3, we find the dimensions of the diffusion constant to be  $L^2t^{-1}$ . Diffusion constants are, accordingly, expressed in square centimeters per second.

Where a relatively concentrated solution on one side of the porous disk diffuses into the pure solvent on the other side and where the compartments on the two sides of the disk are well stirred, we may neglect the differentials in equation 3 and write

$$D = \frac{h}{A} \frac{Q}{tC} \quad (4)$$

where  $h$  is the effective distance through which the solute diffuses,  $A$  is the effective area of the pores of the disk,  $Q$  is the amount of the material that diffuses in time  $t$ , and  $C$  is the concentration of the solution. The ratio  $h/A$  differs for each membrane that is used but is constant for the same membrane regardless of the substance that diffuses through it. This ratio is called the membrane constant; it may be evaluated by the relation:

$$\text{Membrane constant} = \frac{h}{A} = \frac{Dt}{QV} \quad (5)$$

where  $QV$  is the number of cubic centimeters of the initial solution which contains an amount of substance that has diffused through the membrane

<sup>3</sup> J. Northrop and M. L. Anson, *J. Gen. Physiol.* 12, 543 (1928-29); 20, 575 (1937).

<sup>4</sup> J. W. McBain and T. H. Liu, *J. Am. Chem. Soc.* 53, 59 (1931).

<sup>5</sup> J. W. Mehl and C. L. A. Schmidt, *University of California Publications in Physiology* 8, 165 (1937).



in time  $t$ . Since the diffusion constant of a number of substances is known, they may be used to evaluate  $h/A$  of the particular membrane. With this value of  $h/A$ , the diffusion constant of the unknown may be calculated. Actually, it is not necessary to know the absolute amount of the original material which has passed through the membrane, but only the percentage of the amount. If the quantity of material contained in 1 cc. of the concentrated solution is taken as unity and the amount which has diffused is

expressed in this unit, i.e., as the number of cubic centimeters of the concentrated solution containing the quantity that has diffused, equation 3 may be simplified still further to

$$D = \frac{hQ_{cc}}{At} \quad (6)$$

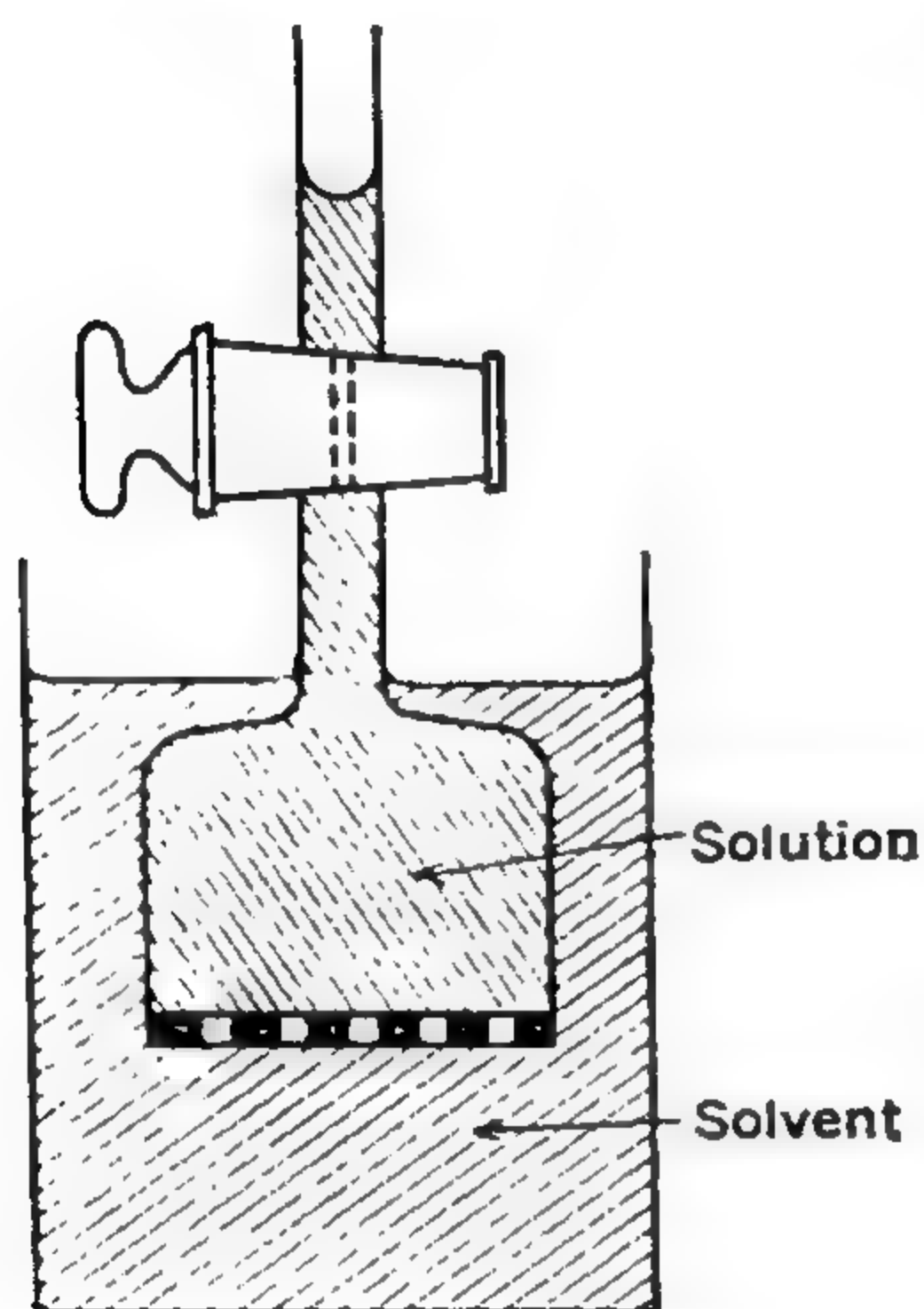


FIG. 2. Diagram of the porous-disk apparatus for the study of diffusion.

where  $Q_{cc}$  is the number of cubic centimeters of the concentrated solution that contains an amount of the substance equal to the amount that has diffused. For example, if it were found in a study on the diffusion of glucose from a 0.1  $M$  solution into pure water that 0.001 mole of glucose had diffused in a given time,  $Q_{cc}$  would evidently be 10; i.e., 10<sub>cc</sub> of the 0.1  $M$  glucose would contain 0.001 mole of glucose. The porous-disk apparatus is diagrammed in Fig. 2.

The porous-disk method is particularly well adapted to the determination of the diffusion constant of small molecules. It also has the tremendous advantage that very dilute solutions of biologically active materials can be used and no knowledge of the absolute concentrations is needed.

The refractometric method depends on the observed fact that the index of refraction of a solution is proportional to the concentration of the solute. An apparatus is used in which the refraction experienced by a beam of light in passing through the boundary between the solvent and the solution can be measured accurately. From this information the change of the concentration gradient with distance from the boundary is determined (see bottom section of Fig. 1). This gradient is measured after appropriate time intervals, and this furnishes us with sufficient information to calculate the diffusion constant.

Diffusion measurements by the refractometric method require that the initial boundary between the solution and the solvent be sharp and well defined. The Neurath<sup>6</sup> diffusion cell appears to meet this requirement

<sup>6</sup> H. Neurath, *Science* 93, 431 (1941).

more conveniently and exactly than any other cell. "The cell consists of two U-shaped stainless-steel blocks which can be slid horizontally past each other by a screw arrangement. Two optically flat rectangular glass windows are pressed against the vertical faces by the top and bottom clamps. The lower compartment, when separated from the top one, is filled with the solution through the left-hand tube; the upper compartment is filled with the solvent through the right-hand tube. After temperature equilibrium, the sharp boundary is formed by bringing the two compartments into vertical alignment. The dimensions of the cell proper are as follows: 1.7 cm. in the direction of the optical axis, 0.5 cm. wide and 5 cm. high."

There are two convenient methods of measuring the displacement of the light after it has passed through the solution-solvent boundary in the diffusion cell. The cylindrical-lens system has already been discussed under electrophoresis (Chapter 9). The other method, which probably is capable of a greater degree of precision than the cylindrical-lens system, although it is a very tedious method, is to photograph an accurately ruled scale through the boundary. The appropriate lens system has to be used to visualize the scale. The scale is also photographed in the absence of the boundary, and the displacement of the scale lines is then measured with a microcomparator. The scale-line displacements are then plotted against the positive and the negative distances from the original boundary. Both the scale-line displacement and the cylindrical-lens system yield a curve of the type shown in the bottom section of Fig. 1.

The problem of obtaining  $D$  from the curve shown in the bottom section of Fig. 1 involves the solution of equation 2 for this constant. Actually, there are four ways of proceeding to do this. Of these ways only the maximum ordinate method will be considered. At  $x$  equal zero, equation 2 becomes

$$\left(\frac{dC}{dx}\right)_{x=0} = y_m = \frac{C}{2\sqrt{\pi Dt}} \quad (7)$$

where  $y_m$  is the maximum ordinate. Since the area under the curve is proportional to the concentration of the solute, we can substitute the area for  $C$  and rearrange the equation to obtain

$$D = \frac{A^2}{4\pi t(y_m)^2} \quad (8)$$

The area  $A$  is determined by graphical integration using a planimeter. We can plot  $t$  in seconds against  $(1/y_m)^2$  and a straight line should be obtained whose slope is  $A^2/4\pi D$ , from which  $D$  can be calculated. The  $D$  so found must be multiplied by the magnification factor of the lens system. The magnification factor is determined by photographing a standard scale

and comparing the known dimensions with those found. There are a number of details of diffusion measurements that we have not considered; for these, and for a splendid treatment of diffusion in general, the reader is referred to the review by Neurath.<sup>2</sup>

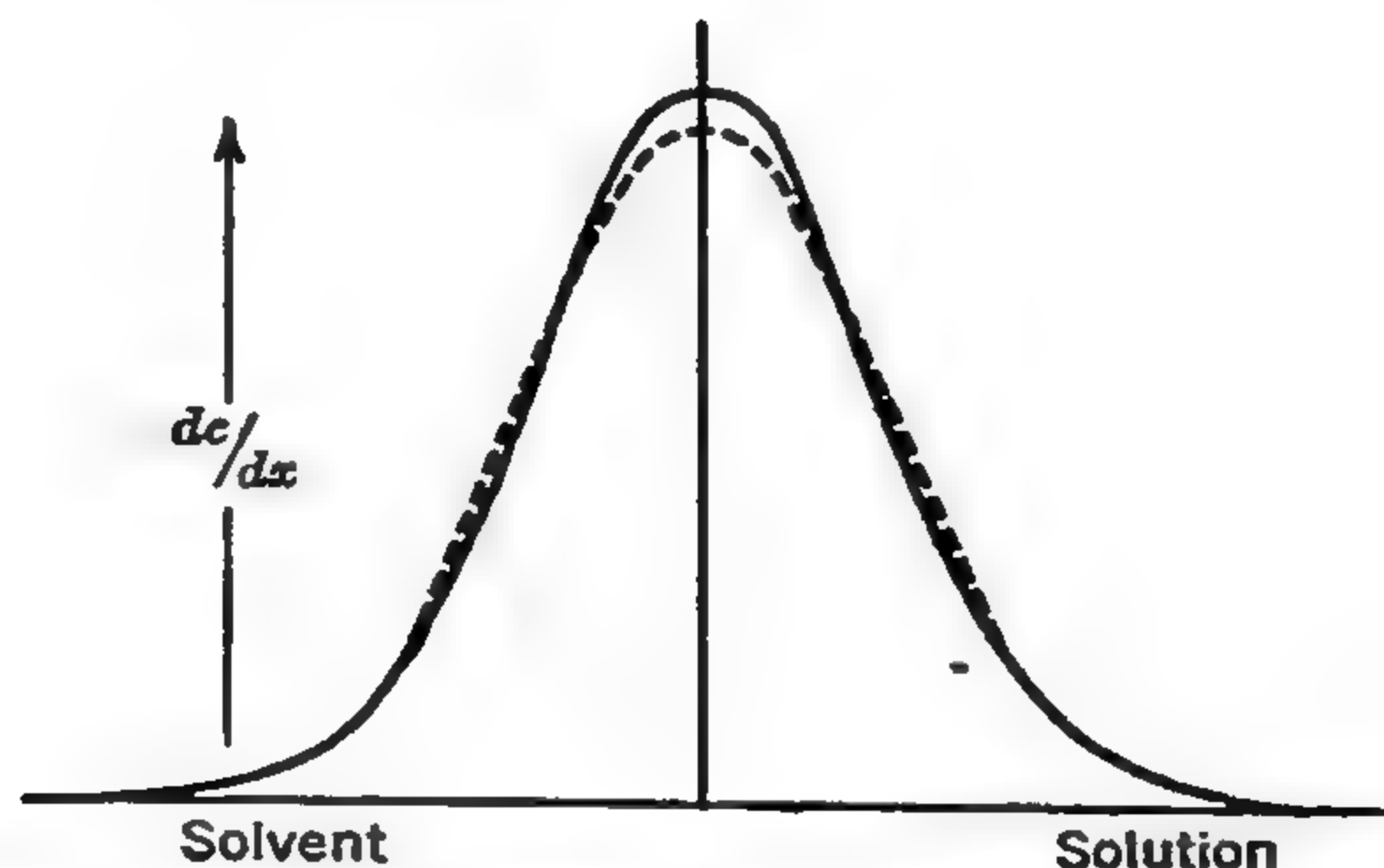


FIG. 3. Comparison of an ideal Gaussian distribution curve with the diffusion curve obtained from measurements on a 6.8 per cent serum albumin solution at pH 7.6 which had been denatured by heating for 30 minutes at 70°C. Polydispersity is most clearly indicated by the difference in maximum ordinate of the ideal curve (broken line) and the experimental curve (solid line). (Neurath.)

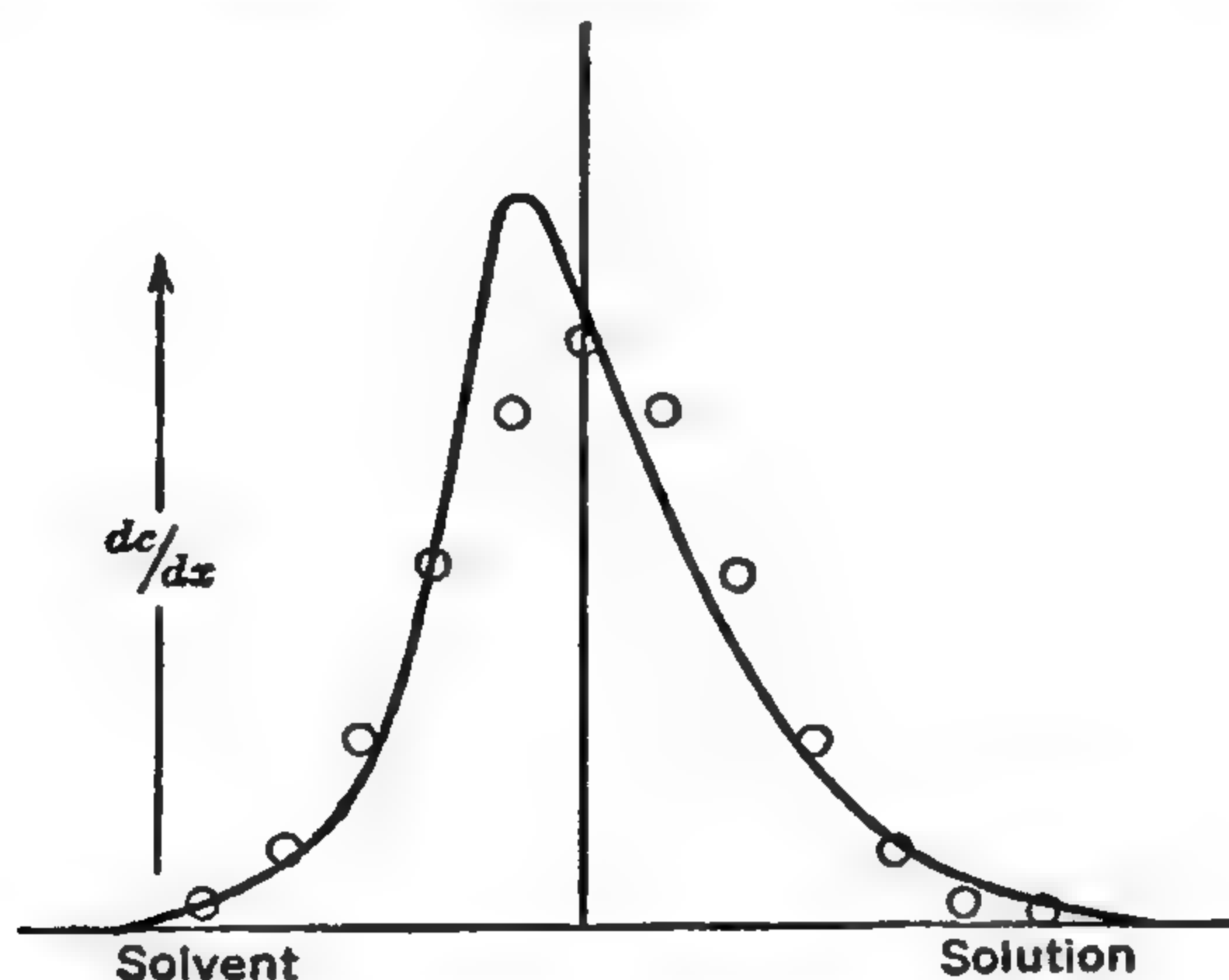


FIG. 4. Comparison of an ideal Gaussian distribution curve with the diffusion curve as obtained from the measurements on a one per cent solution of tobacco mosaic virus protein. The open circles indicate the position of the ideal curve; the solid line refers to the experimental curve plotted in normal coordinates. (Neurath.)

Kegeles and Gosting<sup>7</sup> have described a diffusion method based on the interference-fringe system produced by focusing light through a diffusing boundary. Such an arrangement is said to be accurate and to require very little time. Longworth<sup>8</sup> has reported on the experimental test of this method.

<sup>7</sup> G. Kegeles and L. J. Gosting, *J. Am. Chem. Soc.* 69, 2516 (1947).

<sup>8</sup> I. G. Longworth, *J. Am. Chem. Soc.* 69, 2510 (1947).



The uniformity of the material in respect to particle size can be studied by the refractometric method. It is assumed that the curve showing the displacement of light as a function of the distance from the boundary may be treated as an ideal distribution curve. The identity of the experimental curve with the normal distribution curve indicates monodispersity, whereas deviations from the normal dispersion curve can be interpreted to mean that the material is polydispersed. See Fig. 3.

If there is considerable interaction between the solute particles leading to a certain amount of gel structure in the solution, a skewed curve is obtained when the data are plotted as described above. Such a curve is shown in Fig. 4, which was obtained on a 1 per cent solution of tobacco mosaic virus protein.

### TEMPERATURE INFLUENCES

The diffusion constant measured at a certain temperature can be calculated for any other temperature by the equation<sup>9</sup>

$$D_{T_1} = D_{T_2} \frac{T_1 \eta_{T_2}}{T_2 \eta_{T_1}} \quad (9)$$

where  $D_{T_1}$  and  $\eta_{T_1}$  are the diffusion constant and viscosity of the solvent at temperature  $T_1$ , and  $D_{T_2}$  and  $\eta_{T_2}$  are the corresponding values at temperature  $T_2$ . The temperature is expressed in degrees absolute. For diffusion in water at 20° C. and at 25° C. we have

$$D_{25} = 1.146 D_{20} \quad (10)$$

### CONCENTRATION EFFECTS

The electrostatic charge on colloidal particles can produce anomalous diffusion rates of those particles. In order to circumvent this effect, salts are added to the solvent. It is reported that, if the ionic strength is about 0.1, the electrostatic influence is negligible.

Inasmuch as it is common practice to refer diffusion constants to that in pure water at 20° C. or 25° C., it is necessary to correct for the influence of both the salts and the protein. The correction usually employed is

$$D_x = D_y \frac{\eta_x}{\eta_y} \quad (11)$$

where  $D_y$  is the diffusion constant measured at constant temperature in the solution of viscosity  $\eta_y$ ,<sup>10</sup>  $\eta_x$  is the viscosity of water, and  $D_x$  is the diffusion constant in water.

<sup>9</sup> A. Polson, *Kolloid-Z.* 87, 149 (1939).

<sup>10</sup> M. A. Lauffer, *J. Am. Chem. Soc.* 66, 1188 (1944).

## RESULTS

The diffusion constant of a protein must be known before its molecular weight can be calculated from the rate of sedimentation in the ultracentrifuge. As a consequence of this, the diffusion constants of a large number of proteins have been measured. Reports on these measurements can be found in *The Ultracentrifuge* by Svedberg and Pedersen<sup>11</sup> and in the review by Neurath.<sup>2</sup> Table 1 shows the diffusion constants of a few proteins along with their volume intrinsic viscosities.

TABLE 1

DIFFUSION AND VISCOSITY DATA OF PROTEINS AT 25° C.

(The volume intrinsic viscosities are calculated from the partial specific volumes of the proteins in solution.)

Protein	$D \times 10^7$	$D_0/D$	$[\eta]$
Pepsin B	10.0	1.120	5.17
Egg albumin	8.8	1.158	5.20
Pepsin A	9.2	1.210	5.94
Serum albumin (horse)			
McMeekin fraction	7.4	1.210	4.97
Kekwick fraction	7.0	1.270	5.69
Helix hemocyanin pH 6.67	1.6	1.241	7.05
$\beta$ -Lactoglobulin	8.4	1.258	5.44
Homarus hemocyanin	3.2	1.274	6.39
Seroglycoid (horse)	6.9	1.280	6.73
Amandin	4.2	1.284	6.98
Octopus hemocyanin	1.9	1.383	9.03
Thyroglobulin (pig)	3.0	1.425	9.90
Helix hemocyanin pH 8.6	2.4	1.886	18.73

In Table 1,  $D$  is the measured diffusion constant in square centimeters per second and  $D_0$  is the diffusion constant of a spherical anhydrous molecule of the same molecular weight.

## DIFFUSION OF SPHERICAL PARTICLES

We are here concerned with the diffusion of particles whose volume is much greater than that of the molecules of the solvent.

The force causing diffusion of a dissolved substance is the gradient of the chemical potential for that substance,<sup>12</sup> and, for dilute solutions where the activity and concentration are very nearly equal, the force per mole is

<sup>11</sup> T. Svedberg and K. Pedersen, *The Ultracentrifuge*, Oxford University Press, New York, 1940.

<sup>12</sup> L. Onsager and R. M. Fuoss, *J. Phys. Chem.* 36, 2689 (1932).

$RT dC/dx$ . In a steady state the resisting forces must equal the force causing diffusion and

$$fu = RT \frac{dC}{dx} \quad (12)$$

where  $f$  is the frictional constant per mole and  $u$  is the rate of diffusion. Combining equation 1 with equation 12, we have

$$D = \frac{RT}{f} \quad (13)$$

For spherical particles the frictional constant per mole is Stokes' factor or is  $6\pi r\eta N$ , where  $N$  is Avogadro's number,  $\eta$  is the coefficient of viscosity of the solution, and  $r$  is the radius of the spherical particles. Substituting the frictional constant in equation 13, we have

$$D = \frac{RT}{6\pi r\eta N} \quad (14)$$

which is the equation derived almost simultaneously by Sutherland<sup>13</sup> and by Einstein.<sup>14</sup> Since we are dealing with a spherical molecule, equation 14 may be written as

$$D = \frac{RT}{6\pi\eta N \sqrt[3]{3MV/4\pi N}} \quad (15)$$

Substituting numerical values for the constants in equation 15, we have for spherical molecules in water

$$D^{20^\circ} = \frac{28.82 \times 10^{-6}}{\sqrt[3]{MV}} \text{ square cm. per sec.} \quad (16)$$

$$D^{25^\circ} = \frac{33.06 \times 10^{-6}}{\sqrt[3]{MV}} \text{ square cm. per sec.} \quad (17)$$

where  $D^{20^\circ}$  and  $D^{25^\circ}$  are the diffusion constants of spherical molecules at 20° C. and at 25° C., respectively; where  $MV$  is the cubic centimeters molecular volume.

The diffusion of a spherical molecule is thus inversely proportional to the cube root of the volume of the molecule in solution. If the molecule is hydrated, the diffusion constant will be reduced and the ratio of the

<sup>13</sup> W. Sutherland, *Phil. Mag.* (6) 9, 781 (1905).

<sup>14</sup> A. Einstein, *Ann. Physik* (4) 17, 549 (1905); *Z. Elektrochem.* 14, 337 (1908).



anhydrous diffusion constant to the hydrated diffusion constant will be

$$\frac{D_0}{D_H} = \sqrt[3]{\frac{MV_H}{MV_A}} \quad (18)$$

where  $D_0$  is the diffusion constant of the anhydrous spherical molecule,  $D_H$  is that of the hydrated spherical molecule,  $MV_H$  is the hydrated cubic centimeters molecular volume, and  $MV_A$  is the anhydrous cubic centimeters molecular volume.

Polson<sup>13</sup> has noted that substances whose molecular weight is much below 1000 diffuse at a greater rate than would be expected on the basis of the discussion given above. He concludes that molecules of this molecular weight and less do not exert macroscopic friction against the molecules of the medium but that slipping or inhomogeneous friction takes place.

#### DIFFUSION OF ASYMMETRIC COLLOIDAL PARTICLES

The influence of particle asymmetry on diffusion is somewhat more complicated than the effect of hydration last considered. The problem is a good deal simpler, however, than the relation between particle asymmetry and viscosity which was discussed in the last chapter. Qualitatively, it is easy to see why asymmetry should decrease the diffusion constant of a molecule. The amount of surface exposed to the frictional forces of the medium by an asymmetric particle is greater than that exposed by a spherical particle of the same volume.

Herzog, Illig, and Kudar<sup>14</sup> calculated the frictional constant ( $f$  in equation 13) for a prolate ellipsoid of revolution (cigar-shaped; rotation about major axis of an ellipse) and also for an oblate ellipsoid of revolution (disk-shaped; rotation about minor axis of an ellipse).

Shortly after Herzog, Illig, and Kudar published their derivation, Perrin<sup>15</sup> published an independent derivation of these equations. It is reassuring that Perrin's equations can be reduced to forms identical with those of Herzog, Illig, and Kudar.

The equation for a prolate ellipsoid of revolution can be expressed as

$$\frac{D_0}{D} = \frac{2(1 - b^2/a^2)^{1/2}}{\left(\frac{b}{a}\right)^2 \ln \frac{1 + (1 - b^2/a^2)^{1/2}}{1 - (1 - b^2/a^2)^{1/2}}} \quad (19)$$

where  $D_0$  is the diffusion constant of a spherical particle with the same volume as that of the asymmetric particle,  $D$  is the diffusion constant of

<sup>13</sup> A. Polson, *J. Phys. & Colloid Chem.* 54, 649 (1950).

<sup>14</sup> P. O. Herzog, R. Illig, and H. Kudar, *Z. Physik. Chem.* 167A, 329 (1933).

<sup>15</sup> F. Perrin, *J. phys. radium* 7, 1 (1936).

the asymmetric particle, and  $a$  is the major axis and  $b$  the minor axis of the prolate ellipsoid of revolution.

The corresponding equation for an oblate ellipsoid of revolution is

$$\frac{D_0}{D} = \frac{(1 - b^2/a^2)^{1/2}}{\left(\frac{b}{a}\right)^{3/2} \arcsin \left(1 - \frac{b^2}{a^2}\right)^{1/2}} \quad (20)$$

where  $a$  is the major and  $b$  the minor axis of the oblate ellipsoid of revolution.

Figure 5 shows a graph of  $a/b$  against  $D_0/D$  for prolate and for oblate ellipsoids of revolution, by means of equations 19 and 20.

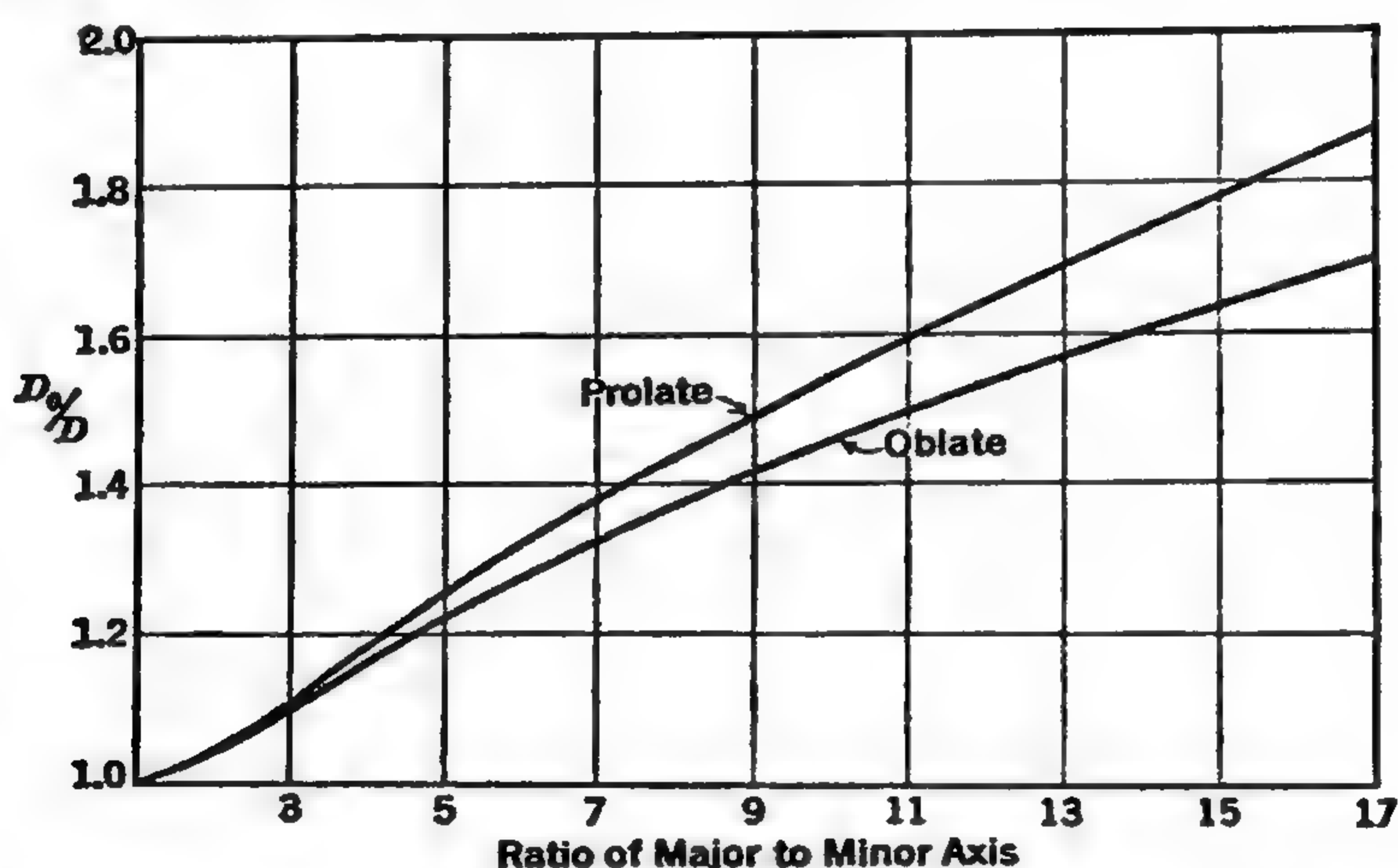


FIG. 5. Relation between asymmetry of prolate and of oblate ellipsoids of revolution and their diffusion constants as calculated from the equations of Herzog, Illig, and Kudar.

Evidently, if one knew the asymmetry of a protein molecule, the hydration could be calculated from diffusion studies, or, if the hydration were known, the asymmetry could be calculated. Since neither the asymmetry nor the hydration is known, we are at an impasse.

It is possible to treat this problem in the following simple and approximate manner.<sup>18</sup>

The values of  $D_0/D$  for various proteins are plotted against the corresponding volume intrinsic viscosities. A line drawn through these points should intersect the  $x$ -axis for  $D_0/D$  equal to unity at 2.5, since we know the volume intrinsic viscosity should be equal to 2.5 for a spherical molecule. We find that this cannot be done, and we conclude that the incorrect value for the specific volume of the proteins has been used. We adjust the specific

<sup>18</sup> H. B. Bull and J. A. Cooper, "Surface Chemistry," edited by Moulton, *Pub. Am. Assoc. Advancement Sci.* No. 21, p. 150 (1943).

volumes of the proteins until the line relating  $D_0/D$  and  $[\eta]$  does intersect the x-axis at 2.5 when  $D_0/D$  is unity. In order to accomplish this an average volume hydration of 28 per cent has been assumed. This value is not in disagreement with other physical measurements. The approximate hydration being known, it is now possible to calculate the approximate asymmetries of the molecules using equations 19 and 20.

The problem of hydration of protein molecules in solution will be discussed again in Chapter 15, on the basis of ultracentrifuge data.

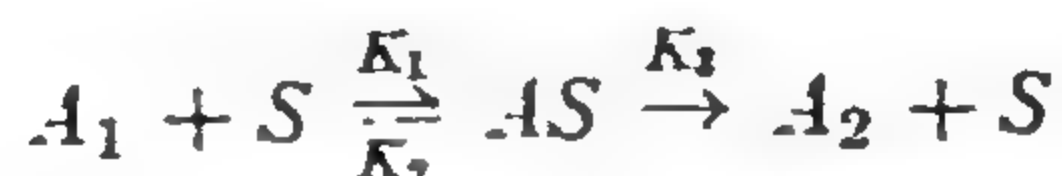
### DIFFUSION THROUGH MEMBRANES

If the pores of the membrane are much larger than the diffusing molecules, the only role the membrane plays is to decrease the cross-sectional area available for diffusion and all molecules diffuse through the membrane at a rate proportional to their diffusion constant and to their concentration gradient across the membrane. As the pores of the membrane become smaller and approach in size that of the diffusing molecules, the interaction between these molecules and the membrane becomes important; the diffusing molecules are adsorbed on the surface of the membrane and penetrate by a process akin to solution in the membrane.

Laidler and Shuler<sup>19</sup> have discussed the kinetics of diffusion through membranes in a clear and useful manner, and a simple version of their theory will be presented.

The rate of diffusion in the liquid phase bathing the membrane is several times greater than the rate of penetration through the membrane, is not a limiting step, and can be neglected in formulating the kinetics of penetration, especially if the liquid phase is well stirred.

The processes occurring at the membrane interfaces are: (1) adsorption on the interface, (2) desorption back into solution, and (3) diffusion into the membrane. Thus we see that the process of penetration bears a close resemblance to the formation and decomposition of an enzyme-substrate complex of the Michaelis-Menten type, described in Chapter 3. At each of the two membrane interfaces the following reactions take place:



where  $A_1$  is the substance in liquid phase and  $A_2$  is the same substance in the membrane.  $S$  is the surface of the membrane. In Chapter 3 we have seen that we may write for such a process

$$v_1 = \frac{VC_1}{K + C_1} \quad (21)$$

<sup>19</sup> K. I. Laidler and K. E. Shuler, *J. Chem. Phys.* 17, 851, 856 (1949).



where  $v_1$  is the rate of penetration of  $A$  through the membrane,  $V$  is the maximum rate of penetration with the membrane surface saturated with  $A$ .  $C_1$  is the concentration of  $A$  in the liquid phase.  $K$  is the so-called dissociation constant but is actually equal to  $(K_2 + K_3)/K_1$ . For a completely symmetrical membrane with both surfaces identical, we may write an equation exactly analogous to equation 21 for the opposite side of the membrane, the net rate of penetration of the substance through the membrane is equal to the difference in rates of penetration in the two directions, and the net rate is

$$v_1 - v_2 = v_n = \frac{KV(C_1 - C_2)}{(K + C_1)(K + C_2)} \quad (22)$$

where  $C_2$  is the concentration on the dilute side of the membrane. When  $K$  is very large in comparison with  $C_1$  and  $C_2$ , there results

$$v_n = \frac{V}{K} (C_1 - C_2) \quad (23)$$

Fick's diffusion law for membranes may be written as

$$v_n = \frac{DA}{d} (C_1 - C_2) \quad (24)$$

where  $A$  is the area of the membrane and  $d$  is its thickness. If the thickness of the membrane is considered constant, it may be incorporated in the diffusion constant to give a permeability constant  $P$ . Equation 24 then becomes

$$v_n = PA(C_1 - C_2) \quad (25)$$

The permeability constant,  $P$ , is thus the number of moles of the substance passing unit cross-sectional area of the membrane in unit time under unit concentration difference. The unit of area customarily chosen by biologists in the study of cell penetration is the square micron; the concentration difference is expressed in moles per liter, and the time in seconds.<sup>28</sup>

Comparison of equation 23 with 25 shows that, if  $K$  is much larger than  $C_1$  and  $C_2$ ,

$$PA = \frac{V}{K} \quad (26)$$

It was noted above that  $K$  is equal to  $(K_2 + K_3)/K_1$ . If  $K_3$  is very much smaller than  $K_2$ ,  $K$  becomes equal to  $K_2/K_1$  which is equal to the reciprocal of adsorption coefficient on the membrane surface. It is also propor-

<sup>28</sup> H. Davson and J. F. Danielli, *The Permeability of Natural Membranes*, Cambridge University Press, 1943.

tional to the reciprocal of the distribution coefficient of the diffusing molecules between a liquid membrane and the solvent. Thus, when  $K_2$  is much larger than  $K_3$ , the rate of penetration becomes directly proportional to the distribution coefficient of the diffusing molecules between the membrane and the solvent.

It is possible to apply the theory of the activated state to the penetration of membranes by diffusing molecules, and under favorable conditions to calculate the free energy, the heat energy, and the entropy of activation for the process.<sup>21</sup>

### PENETRATION INTO LIVING CELLS

This discussion can be divided into three parts: (1) water penetration, (2) electrolyte penetration, and (3) penetration of unionized organic molecules.

The passage of water across cell membranes apparently involves no active mechanism, and water is transferred into and out of cells in response to differences in osmotic pressure. The subject has been reviewed by Lucke and McCutcheon<sup>22</sup> and by Lucke.<sup>23</sup> Laidler and Shuler<sup>19</sup> have also given a stimulating discussion of the rate of transport of water across membranes.

Not only do living cells swell or shrink in response to a decrease or increase of the osmotic pressure of the external medium but also the relation is, in general, a quantitative one:

$$P_e(V_e - b) = P_0(V_0 - b) \quad (27)$$

where  $P_e$  is the known osmotic pressure of the external medium,  $V_e$  is the expected equilibrium volume of the cell,  $P_0$  is the original osmotic pressure of the external medium,  $V_0$  is the original volume of the cell, and  $b$  is a constant that is in the nature of a correction for the volume occupied by the osmotically inactive materials in the cell. The value of  $b$  varies considerably with different varieties of cells. Thus for sea-urchin eggs it is about 12 per cent of the initial total volume of the cell, whereas for mammalian red blood cells it is much larger—about 45 per cent.

The rate of uptake of water by living cells in response to osmotic changes varies from one type of cell to another, but it is usually surprisingly rapid, complete adjustment frequently requiring only a few minutes. The osmotic pressure of the external medium can be controlled by electrolyte addition or dilution, and the osmotic response of the cell is still in accord with equation 27. This indicates that the rate of water penetration through cell membranes greatly exceeds that of electrolyte penetration.

<sup>21</sup> B. J. Zwolinski, H. Eyring, and C. E. Reese, *J. Phys. & Colloid Chem.* 53, 1426 (1949).

<sup>22</sup> B. Lucke and M. McCutcheon, *Physiol. Revs.* 12, 68 (1932).

<sup>23</sup> B. Lucke, *Cold Spring Harbor Symposium Quant. Biol.* 8, 123 (1940).

The penetration of ions into living cells was discussed in Chapter 4, and it was noted that ionic penetration is frequently associated with an active mechanism involving cellular metabolism.

Experimental data seem to indicate that non-electrolytes usually penetrate living cells by simple diffusion in which the only significant concentration gradient is that across the membrane itself. Sometimes a non-electrolyte apparently does involve an active mechanism. For example,

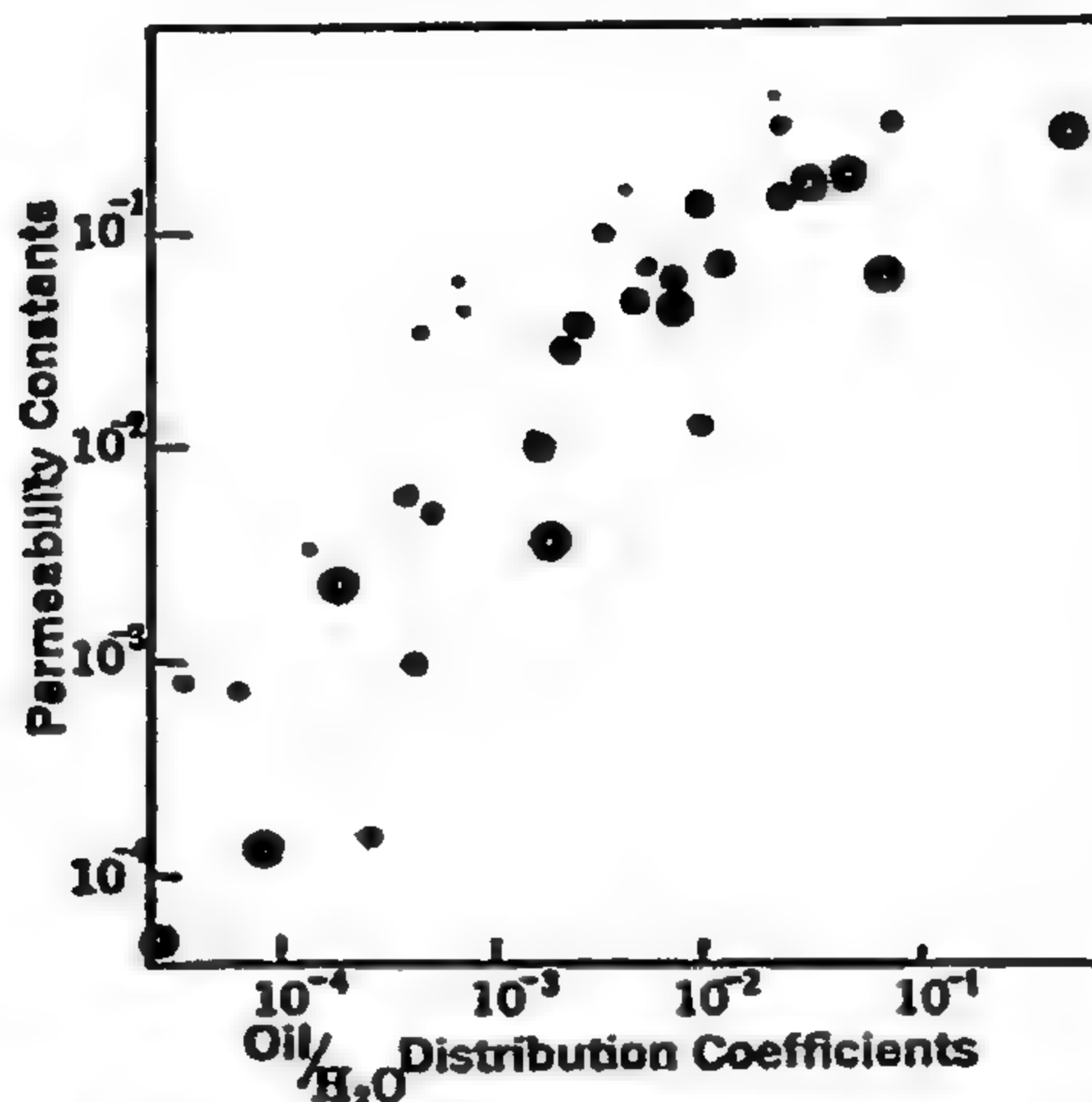


FIG. 6. Permeability of the cells of *Chara ceratophylla* to various non-electrolytes. Ordinate indicates the permeability constants (centimeters per hour). The abscissa gives the oil-water distribution of the substances. The relative sizes of the circles indicate the relative sizes of the molecules. (Collander.)

the uptake of glucose from the intestines of mammals seems to depend on such an active mechanism.

Many years ago Overton<sup>24</sup> found that a general parallelism existed between the rate of penetration of various organic substances into living cells and their lipid-water distribution coefficients, and he concluded that the membrane of living cells was fat-like in nature. A by-product of this penetration theory was the lipid theory of narcosis. It was found that there also existed a parallel between the oil-water distribution of narcotizing substances and the concentration of these substances required to narcotize such animals as tadpoles.

Collander<sup>25</sup> has reported a study of the penetration of 45 non-electrolytes into the alga *Chara ceratophylla*. He also determined the distribution of these electrolytes between olive oil and water. Shown in Fig. 6 is a plot of

<sup>24</sup> E. Overton, *Pflügers Arch. ges. Physiol.* 92, 115 (1902).

<sup>25</sup> R. Collander, *Trans. Faraday Soc.* 33, 985 (1937).



the permeability constants of these compounds against their oil-water distribution coefficients. The relative sizes of the circles indicate the relative sizes of the penetrating molecules. Clearly, there is a close correlation between oil-solubility of a substance and its permeability constant. However, the smallest molecules penetrate faster than would be expected on account of their oil-solubility alone.

Collander<sup>26</sup> has published additional information on the oil-water distribution coefficients of various substances.

### ROTARY DIFFUSION CONSTANT

Suppose that all the particles in a suspension are oriented in the direction of some external force and the force is suddenly removed. Brownian motion of the particles would then lead to random orientation. The relaxation time  $\tau$  is a measure of the time required for the particles to assume random distribution of orientation and is

$$\tau = \frac{\text{Resisting forces}}{2kT} \quad (28)$$

where  $k$  is Boltzmann constant and  $T$  is the absolute temperature. The resisting forces will be a function of both the size and the shape of the particles, and, accordingly, a determination of the relaxation time is capable of yielding valuable information about suspended particles. The rotary diffusion constant  $\theta$  is a measure of the tendency of a particle to rotate, and the rotary diffusion constant is equal to  $1/2\tau$ . A spherical particle will have only one relaxation time and one rotary diffusion constant because the Stokes' resisting forces are equal to  $8\pi\eta r^2$ , which can be substituted in equation 28 to yield the relaxation time of a rigid sphere. For ellipsoids, the relation is more complex because the resisting forces cannot be so simply treated and, in general, three relaxation times will be required to describe the motion about the three axes.<sup>27</sup>

The relaxation times of molecules in solution can be determined by stream birefringence and by a study of the dielectric constant as a function of the frequency.

### STREAM BIREFRINGENCE

The velocity gradient in a flowing liquid tends to orient suspended particles. There is a continual rotation of such particles in a flow gradient: a prolate ellipsoid rotates most rapidly when the major axis is perpendicular to the streamlines and most slowly when this axis is parallel to the stream-

<sup>26</sup> R. Collander, *Acta Physiol. Scand.* 13, 363 (1947).

<sup>27</sup> F. Perrin, *J. phys. radium* (7) 5, 497 (1934).

lines. If the major axis is very much greater than the minor axis, the particle spends nearly all its time with the major axis parallel to the streamline. The degree of orientation increases with increasing velocity of flow. The oriented particles interrupt the streamlines of flow less than unoriented particles, and, accordingly, the viscosity of such a solution decreases with increasing rate of flow: this situation has already been noted in red blood cells.

A convenient way of measuring the degree of orientation in a flow gradient is by a study of the flow or stream birefringence of the solution.

As is well known, ordinary light vibrates in all planes. If such light is passed through a calcite crystal in the proper direction, the beam of light is broken into two beams. This can be demonstrated by viewing an object through a calcite crystal. Upon rotation of the calcite crystal, a double vision is observed to appear. It will be discovered that there is one direction in which light can be passed through a calcite crystal without being broken into two beams. This direction corresponds to the optic axis of the crystal. Calcite, like a number of other crystals, is anisotropic, the index of refraction being different in two directions through the crystal. Both beams of light emerging from such a crystal are plane polarized, and the planes of vibration of the two beams are at right angles to each other. The magnitude of double refraction is measured by the difference between the index of refraction of the ordinary ray ( $n_o$ ) and that for the extraordinary ray ( $n_e$ ). Light whose plane of vibration is parallel to the optic axis is called the extraordinary ray; and light whose plane of vibration is perpendicular to it is known as the ordinary ray. If  $n_e > n_o$ , the double refraction is positive; if  $n_e < n_o$ , it is negative.

Double refraction of an object can be detected by placing it between two crossed Nicol prisms. Light passing through the first Nicol (the polarizer) is plane polarized. When it enters the anisotropic object the plane-polarized beam is broken into two rays, one vibrating parallel to the optic axis of the object and the other at right angles to it. If white light has been used, the field, as viewed through the second Nicol (the analyzer), is seen to be beautifully colored and there will be no position of the second Nicol at which all the light can be extinguished. Since the velocity of light is inversely proportional to the index of refraction and since each wavelength of light has its own index of refraction through a medium, the velocities of the various wavelengths in the two beams of light through the double refracting object are all different, so that, on emergence of the light, some of the wavelengths will differ in phase and their intensity will be diminished, whereas others will be in phase and will be reinforced. The net effect is that the object appears highly colored and the color varies as the analyzer is rotated. Light emerging from the anisotropic object is said to be elliptically polar-



ned because the amplitudes of the waves of the two beams of plane-polarized light, when combined, describe an ellipse.

If a suspension of long asymmetric particles, such as those of vanadium pentoxide, is placed between crossed Nicols, the field will be completely dark. If, however, streaming motion is produced in the suspension, the suspension becomes double refracting as is evidenced by a light field which persists as long as the suspension is in motion. This type<sup>25</sup> of birefringence is known as stream double refraction or stream birefringence.

Double refraction of flow may be due to the orientation of either optically isotropic or optically anisotropic particles. Double refraction due to orientation of optically isotropic particles varies with the index of refraction of the medium and becomes zero when the index of refraction of the medium is made the same as that of the particles. The stream double refraction of optically anisotropic particles also varies with the index of refraction of the medium, but it never becomes zero: it passes through a minimum.

There are two general experimental approaches to the study of stream double refraction. One method used by Laufer and Stanley<sup>26</sup> and by Laufer<sup>27</sup> in their study of the stream double refraction of solutions of tobacco mosaic virus protein consists simply in observing a capillary tube between crossed Nicols. The solution was forced back and forth in the capillary by means of a pump arrangement. The observation was carried out with a polarizing microscope. Such a microscope is equipped with a polarizer below the object and an analyzer above the eyepiece. Stream double refraction was measured by the lightness of the field. The intensity of light, which is proportional to the stream double refraction, was measured with a photoelectric cell. The tobacco mosaic virus protein, being rod-like with considerable asymmetry, gives a very pronounced stream double refraction which in the more dilute solutions was found to be proportional to the protein concentration. Unfortunately, not very much information can be obtained by this simple method of study. About all one can really do with it is to show the presence of stream double refraction and to investigate the influence of concentration.

The other method is better adapted to quantitative studies. In this method two concentric cylinders are employed: the inner one is stationary; the outer one contains the suspension and is rotated. The velocity of flow of the liquid at the boundary of the inner cylinder is zero; that at the boundary of the outer cylinder is equal to the velocity of motion of the outer cylinder. The velocity of flow thus varies from zero to some finite value, and the velocity gradient is uniform in the direction normal to the surface

<sup>25</sup> H. Freundlich, F. Stapelfeldt, and H. Zocher, *Z. Physik. Chem.* 114, 161, 190 (1924-25).

<sup>26</sup> M. A. Laufer and W. M. Stanley, *J. Biol. Chem.* 123, 507 (1938).

<sup>27</sup> M. A. Laufer, *J. Phys. Chem.* 42, 935 (1938).



of the inner cylinder. The solution between the two cylinders is viewed between crossed Nicol prisms or polaroids with monochromatic light. The liquid is isotropic and dark when at rest. Upon rotation of the outer cylinder (or inner one, for that matter), stream double refraction is observed. Figure 7 is a diagrammatic representation of the state of affairs.

The dark brushes or arms of the cross mark the regions where the optic axis of the flowing liquid is parallel to the plane of polarization of either the analyzer or the polarizer.  $\psi$ , the angle of isocline, corresponds to an angle

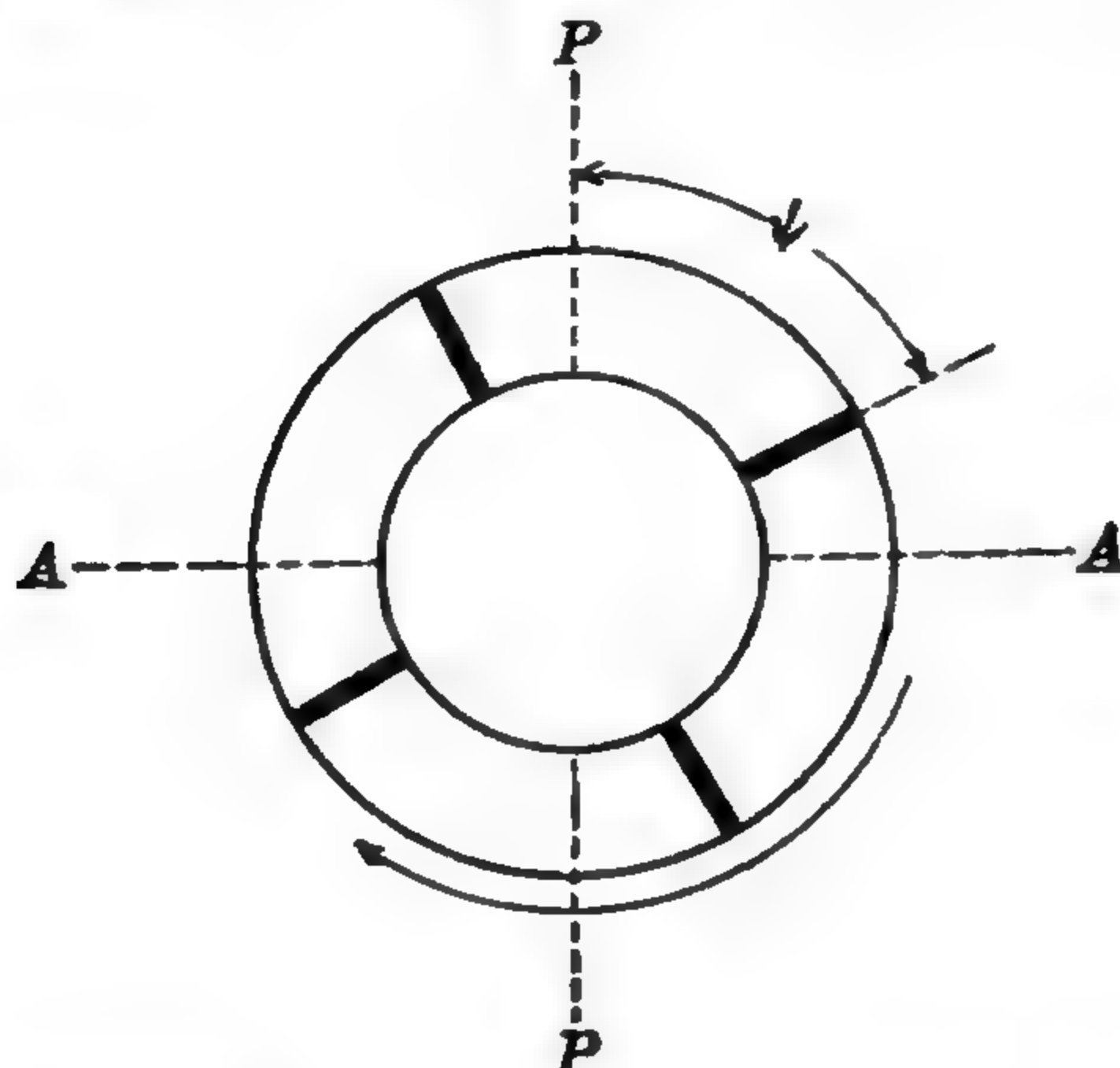


FIG. 7. The cross of isocline which is due to the orientation of particles in the streamlines between a rotating and a stationary cylinder.  $PP$  and  $AA$  are the planes of vibration of light transmitted by the polarizer and analyzer, respectively.

$90^\circ - \psi$  to the streamline. The angle of isocline is always the larger of the two angles between the cross and the plane of polarization of either the analyzer or the polarizer. The angle between the optic axis and the streamlines—known as the extinction angle,  $\chi$ —is always between  $0^\circ$  and  $45^\circ$ . Thus, the extinction angle is the complement of the angle of isocline, the sum of the extinction angle and the angle of isocline being  $90^\circ$ . Either of these two angles may be used to define the position of the optic axis. The other type of measurement is the determination of the double refraction ( $n_e - n_0$ ). This is done by means of a compensator, which is a birefringent plate with a phase difference of a quarter wavelength between the two components vibrating parallel and perpendicular to its optic axis. This compensator is set with its optic axis perpendicular or parallel to  $PP$  or  $AA$  (Fig. 7), and the analyzer is rotated until the light is extinguished. This angle of rotation is a measure of ( $n_e - n_0$ ).

At very low velocity gradients, the degree of orientation is small, and the most favored orientation is  $45^\circ$  to the streamlines; as the velocity gradient increases, the orientation becomes more pronounced and the angle of most

favorable orientation moves towards the streamlines.  $\chi$  is the angle between the streamlines and the optic axis in the flowing liquid. Thus,  $\chi$  tends from  $45^\circ$  at very low to  $0^\circ$  at very high gradients. It has been found possible to relate the relaxation time (rotary diffusion constant) to the extinction angle and at lower flow gradients

$$\tan 2\chi = \frac{6\theta}{G} \quad (29)$$

where  $G$  is the flow gradient and is equal to  $2\pi r N / 60d$ , in which  $r$  is the mean radius of the cylinders,  $N$  is the speed of the rotor in r.p.m., and  $d$  is the gap width.

The relation between the rotary diffusion constant and the axial ratios of a prolate ellipsoid of revolution is, provided that the ratio of the major axis to the minor axis is greater than 5,

$$\theta = \frac{1}{2\tau} = \frac{3kT}{16\pi\eta a^3} \left( 2 \ln \frac{2a}{b} - 1 \right) \quad (30)$$

The rotary diffusion constant is relatively insensitive to variation in the logarithmic term; the approximate value of  $\ln 2a/b$  can be found from viscosity, etc.; and the rotary diffusion constant varies essentially inversely with the cube of the length of the molecule. Stream birefringence of protein solutions have been made by a number of workers.<sup>21, 22, 23, 24</sup>

Robinson<sup>25</sup> reports an interesting study of the stream double refraction of tobacco mosaic virus protein. This study was paralleled by an investigation of the viscosity of the solutions. Robinson used a Couette viscometer, which consists, as we have seen, of two concentric cylinders. The inner cylinder is stationary and suspended by a fine wire; the outer one contains the suspension and is rotated. Robinson viewed the solution between the inner and outer cylinders with crossed Nicol prisms. He was thus able to make simultaneous optical and viscosity measurements. He found that, with increasing speeds of rotation of the outer cylinder, the stream double refraction increased. At the same time the viscosity decreased. The increased double refraction with increasing speeds is evidence of increased orientation of the particles of tobacco mosaic virus. Increasing orientation evidently leads to a decrease in viscosity. This is to be anticipated, since there is less interruption of the flow lines by the orientated particles.

<sup>21</sup> J. F. Foster and J. T. Edsall, *J. Am. Chem. Soc.* 67, 617 (1945).

<sup>22</sup> J. T. Edsall, J. F. Foster, and H. Scheinberg, *J. Am. Chem. Soc.* 69, 2731 (1947).

<sup>23</sup> J. T. Edsall and J. F. Foster, *J. Am. Chem. Soc.* 70, 1860 (1948).

<sup>24</sup> E. Fredericq, *Bull. soc. chim. Belges* 56, 223 (1947).

<sup>25</sup> I. R. Robinson, *Proc. Roy. Soc. (London)* A170, 519 (1939).

## ROTARY DIFFUSION CONSTANT AND THE DIELECTRIC CONSTANT

In Chapter 1 was given a short discussion of the dielectric constant and dipole moments. Here we wish to outline the problem of the variation of the dielectric constant with frequency and show how the relaxation time can be obtained from such studies.

Evidently, if the frequency of alternations of the charge on the condenser plates is progressively increased, a frequency will be reached at which neither the solute nor the solvent molecules will be able to rotate with sufficient rapidity to respond to the alternations. If the molecules of the solute are

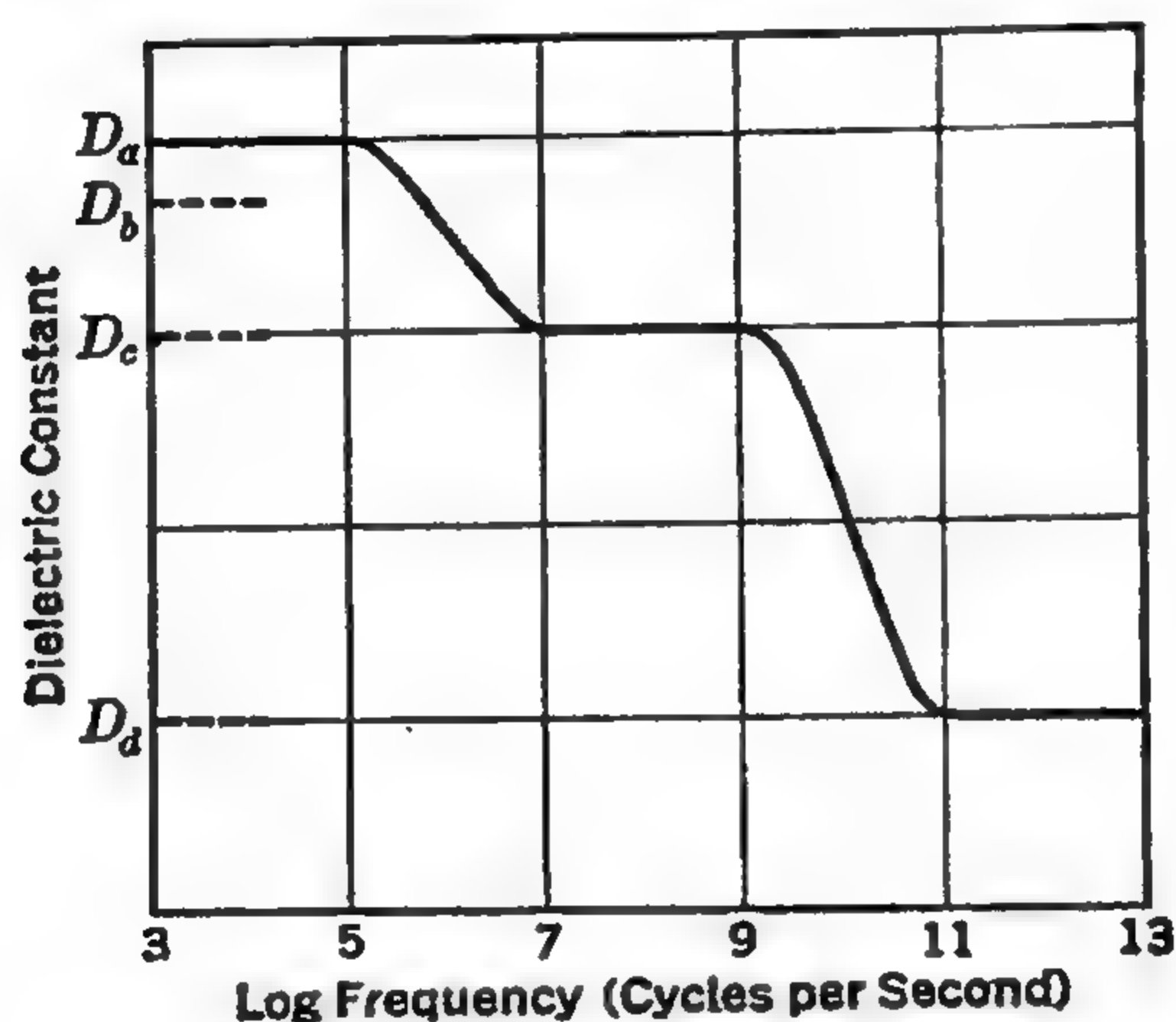


FIG. 8. Anomalous dispersion curve of the dielectric constant of an aqueous protein solution (diagrammatic).

enough larger than those of the solvent and the dielectric constant of the solution is plotted against the logarithm of the frequency, a curve such as is diagrammatically shown in Fig. 8 must be obtained. Such a curve is known as an anomalous dispersion curve.

In Fig. 8,  $D_a$  is the dielectric constant of the solution;  $D_b$  is the dielectric constant of the pure water;  $D_c$  is the dielectric constant of the solution at a frequency which is too great for the protein molecules to respond to the alternations. Note that  $D_c$  is less than  $D_b$ ; the solute molecules are inert at this frequency and occupy a certain volume so that there is less water per unit volume solution than for pure water.  $D_d$  is the dielectric constant of the solution at such a high frequency that neither the solute nor solvent molecules can respond to the alternations in the sign of the charge. At this frequency only the atomic and electronic factors of polarization are operating.

It is clear that there should exist a relation between molecular size and the critical frequency at which dispersion of the dielectric constant occurs. This is indeed true, and the mathematical relations are outlined below.



The time of relaxation is a measure of the time required for the orientated dipoles to revert to random distribution if they were all oriented at zero time. The time of relaxation is related to the frequency of the field and to the dielectric constant as follows:

$$\tau = \frac{D_c + 2}{D_a + 2} \cdot \frac{1}{2\pi\nu_c} \quad (31)$$

where  $\tau$  is the relaxation time and  $\nu_c$  is the critical frequency which is defined as that frequency at which

$$D = \frac{D_a + D_c}{2} \quad (32)$$

Since it is possible to evaluate the relaxation times of molecules by means of dielectric constant studies, it is possible to use this method for the calculation of the dimensions of the molecules.<sup>36</sup>

### PROBLEMS AND QUESTIONS

1. Compare the advantages and disadvantages of the refractometric and porous-disk method for the determination of diffusion constants.

2. The measured diffusion constant of a certain protein is  $9.3 \times 10^{-7}$  sq. cm. per second at 25° C. The molecular weight of this protein is 16,500, and its specific volume is 0.75 cc. Estimate the ratio of major to minor axis of the protein molecule, assuming the molecule to be an anhydrous prolate ellipsoid of revolution. *Ans.:  $a/b = 9.6$*

3. Calculate the rotary diffusion constant of the protein in Problem 2.

*Ans.:  $\Theta = 2.97 \times 10^6 \text{ sec}^{-1}$*

\* E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids, and Peptides*, Reinhold Publishing Corp., New York, 1943.

## THE ULTRACENTRIFUGE

It is neither necessary nor desirable to enter into a detailed discussion of the ultracentrifuge. The book on the ultracentrifuge by Svedberg and Pedersen<sup>1</sup> supplies a wealth of information. In addition, there have been several reviews by authorities in the field. One of these reviews is by Pickels.<sup>2</sup>

The rate of sedimentation of suspension under the influence of gravity had been used by a number of early workers to estimate the particle size of suspensions. Stokes' formula relating the rate of fall of a spherical particle to its radius was employed to give an approximation of particle size. Indeed, Oden<sup>3</sup> developed his method of tangential intercepts to obtain information about the particle-size distribution. In 1923 Svedberg and Nicols<sup>4</sup> employed for the first time a centrifuge to increase the gravity force and to speed up the rate of sedimentation for the purpose of measuring particle sizes. The pioneer work has been followed by a gradual development in the technique of ultracentrifugation until today this instrument is the single most powerful tool for physical research on proteins and on other colloidal molecules.

## METHODS

The ultracentrifuge rotates at very great speeds and necessitates very precise experimental control. The oil-driven turbine type employed by Svedberg as well as by a few other workers is very expensive—so expensive in fact that its cost is prohibitive for most laboratories. Beams and co-workers<sup>5</sup> have developed an air-driven ultracentrifuge which is not so elaborate as the Svedberg type and of which the cost of construction is less.

<sup>1</sup> T. Svedberg and K. O. Pedersen, *The Ultracentrifuge*, Oxford University Press, New York, 1940.

<sup>2</sup> E. G. Pickels, *Biophysical Research Methods*, edited by Uber, Interscience Publishers Inc., New York, 1950.

<sup>3</sup> S. Oden, *Kolloid-Z.* 18, 33 (1916).

<sup>4</sup> T. Svedberg and J. B. Nicols, *J. Am. Chem. Soc.* 45, 2910 (1923)

<sup>5</sup> J. W. Beams, F. W. Linke and P. Sommer, *Rev. Sci. Instruments* 9, 245 (1938).

McBain <sup>6</sup> has described a very simple and relatively inexpensive type of ultracentrifuge which, however, cannot be expected to have the flexibility and accuracy of the more expensive instruments. Pickels <sup>7</sup> has constructed a complete apparatus that is electrically driven and is commercially available.

With the exception of the McBain opaque ultracentrifuge which has a mechanical device for immobilizing the centrifuged material, some optical system is needed to visualize the boundary of the sedimentating material. The lens systems employed are modifications of those used in the study of electrophoresis and of diffusion. These systems have already been described.

With any type of ultracentrifuge or of optical system the determination of the molecular weight can be carried out in one of two ways. The speed of the ultracentrifuge can be made very large and the rate of sedimentation measured. Such a determination can usually be completed in a few hours. The other type of determination consists in rotating the centrifuge at a more moderate speed and allowing the solution to reach an equilibrium, the solution being more concentrated towards the outer portion of the centrifuge tube. In rate measurements, forced diffusion occurs under the influence of the centrifugal gravity field, and the net force acting on a mole of particles at a distance  $X$  from the axis of rotation is simply the difference between the centrifugal weight and the buoyancy exerted by the displaced medium, and

$$\text{Force acting} = M\omega^2 X - M\omega^2 X \bar{V}_2 \rho \quad (1)$$

or

$$\text{Force acting} = M(1 - \rho \bar{V}_2)\omega^2 X \quad (2)$$

where  $M$  is the gram-molecular weight,  $\omega$  is the angular velocity in radians per second,  $X$  is the distance from the axis of rotation,  $\bar{V}_2$  is the partial specific volume of the particles, and  $\rho$  is the density of the solution. In a steady state, the net centrifugal force must equal the resisting forces of the medium. These resisting forces will vary with the hydration and the asymmetry of the particles as well as with the velocity of migration of the particles, and

$$\text{Resisting forces} = f_s \frac{dX}{dt} \quad (3)$$

where  $f_s$  is the frictional force per mole per unit speed. Equating the net centrifugal force and the frictional forces, we have

$$M(1 - \rho \bar{V}_2)\omega^2 X = f_s \frac{dX}{dt} \quad (4)$$

<sup>6</sup> J. W. McBain, *Chem. Revs.* 24, 289 (1939).

<sup>7</sup> E. G. Pickels, Specialized Instruments Corp., Belmont, Calif.



The expression  $\frac{dX/dt}{\omega^2 X}$  is the sedimentation rate per unit field of force and is denoted by  $S$ . Accordingly,

$$M = \frac{f_s S}{(1 - \rho \bar{V}_2)} \quad (5)$$

Diffusion involves exactly the same frictional forces as sedimentation in the ultracentrifuge; accordingly

$$D = \frac{RT}{f_s} \quad (6)$$

where  $R$  is expressed in ergs per degree per mole and is equal to  $8.315 \times 10^7$ . Equation 5 then becomes

$$M = \frac{RTS}{D(1 - \rho \bar{V}_2)} \quad (7)$$

We see from equation 7 that, in order to calculate the molecular weight from rate-sedimentation studies, we must know  $S$  the sedimentation per unit field of force,  $D$  the diffusion constant,  $\rho$  the density of the solution, and  $\bar{V}_2$  the partial specific volume of the particles.

After the molecular weight has been determined, the frictional force for a spherical molecule of the same molecular volume can be calculated by means of the Sutherland-Einstein diffusion equation. This frictional force, known as the frictional coefficient, is denoted by  $f_0$ . The ratio  $f/f_0$  was at one time known as the asymmetry ratio. It must be realized, however, that although  $f/f_0$  is related, in a complicated way, to the molecular asymmetry, the relation is not a direct one. It can be seen that

$$\frac{f}{f_0} = \frac{D_0}{D} \quad (8)$$

and  $f/f_0$  can be substituted for  $D_0/D$  in the equations of Herzog, Illig, and Kudar to estimate the molecular asymmetry. Molecular hydration confuses this relation between  $f/f_0$  and asymmetry as it does in diffusion.

The other way of proceeding in a determination of the molecular weight, as was indicated above, is to allow an equilibrium condition to be established and to measure the concentration at distances  $X_1$  and  $X_2$  from the axis of rotation. The measurement of the concentration has to be made by optical means. At equilibrium we have

$$M = \frac{2RT \ln C_2/C_1}{(1 - \rho \bar{V}_2)\omega^2(X_2^2 - X_1^2)} \quad (9)$$

Note that equation 9 is in the form of a Boltzmann distribution, i.e.,

$$C_2 = C_1 e^{M\psi/RT} \quad (10)$$

where  $\psi$  is a "gravitational potential" and is equal to  $(1 - \rho\bar{V}_2)\omega^2(X_2^2 - X_1^2)$ .

The equilibrium-sedimentation is in a sense equivalent to an osmotic-pressure determination. Its theoretical background is somewhat clearer than that of the rate-sedimentation method. The time required for equilibrium to be reached, however, is rather extended, and from an experimental point of view the equilibrium-sedimentation method is not as satisfactory as the rate-sedimentation method.

### PARTIAL SPECIFIC VOLUME

At various points in this book we have used the term partial specific volume. As a knowledge of the partial specific volume is necessary to complete the calculation of the molecular weights from ultracentrifugation data, it seems appropriate to discuss the meaning and measurement of partial specific volumes at this time.

The partial specific volume of a solute may be defined as that volume increase suffered by a very large volume of solution upon the addition of 1 gram of solute. A more useful way of expressing the partial specific volume is

$$\text{Partial specific volume } (\bar{V}) = \frac{dV}{dw} \quad (11)$$

where  $dV$  is the infinitesimal increase in the volume of a solution due to the addition of an infinitesimal weight of the solute.

The partial specific volume may be determined experimentally by plotting the weights of the contents of a pycnometer for several concentrations against the weight concentration of the solute expressed as weight fraction. The weight fraction is 0.01 times the weight per cent concentration. The best smooth curve is drawn through the points of the plot and the slope of the line determined. This gives  $dw/dx_2$ , where  $w$  is the weight of the contents of the pycnometer and  $x_2$  is the weight fraction of the solute. The partial specific volume can then be calculated by the equation

$$\bar{V}_2 = \frac{1}{\rho} - \frac{(1 - x_2)}{\rho w} \frac{dw}{dx_2} \quad (12)$$

where  $\rho$  is the density of the solution.

It will be found for many dilute solutions, particularly those of proteins, that the partial specific volume is independent of the concentration. In this event, the apparent partial specific volume may be used in place of the

true partial specific volume. The apparent partial specific volume of the solute is defined as

$$\bar{V}_a = \frac{V - w_1 v_1}{w_2} \quad (13)$$

where  $V$  is the total volume of the solution,  $w_1$  is the weight of the solvent,  $v_1$  is the specific volume of the solvent, and  $w_2$  is the weight of the dissolved solute. The apparent partial specific volume of the solute can be determined directly from a density measurement, by means of a pycnometer. The apparent partial specific volume  $\bar{V}_a$  is related to the partial specific volume  $\bar{V}_2$ , as a ratio of finite increments to the corresponding differential coefficients.

The partial specific volume is a measure of the volume of solvent displaced by the solute. It does not represent and is not directly related to the actual volume occupied by the hydrated solute molecules in solution.

#### HYDRATED VOLUME IN SOLUTION

One of the main problems to be settled in connection with the actual volume occupied by the dissolved, hydrated molecule is whether water molecules penetrate the molecular structure of the protein or other biopolymers. If water cannot penetrate the protein molecule, then the amount of water that can be bound will be limited by the surface area of the protein molecule. For example, a spherical protein molecule with a molecular weight of 45,000 could accommodate about 0.45 gram of water per gram of protein if the water is packed in a monolayer around the spherical particle. This would mean that about 37 per cent of the volume of the molecule in solution would be water. As noted in Chapter 14, the average volume hydration for a number of proteins as obtained from a combination of viscosity and diffusion data is about 28 per cent. This result seems to indicate that water does not penetrate most native protein molecules when they are dissolved in water. X-ray diffraction studies on methemoglobin crystals<sup>8</sup> as well as on tobacco mosaic protein<sup>9</sup> seems to show that water does not penetrate these protein molecules.

It is possible that, in the presence of urea, water can penetrate protein molecules. Urea is a denaturing agent for proteins and likewise an excellent solvent for them. Consider the results of Neurath and Saum,<sup>10</sup> who studied the increase of viscosity and the decrease of the diffusion constant of horse serum albumin as a function of the urea concentration. It is possible to calculate the volume occupied by the serum albumin molecules by

<sup>8</sup> J. Boyes-Watson and M. F. Perutz, *Nature* 151, 714 (1943).

<sup>9</sup> J. D. Bernal and I. Fankuchen, *J. Gen. Physiol.* 25, 111 (1941).

<sup>10</sup> H. Neurath and A. M. Saum, *J. Biol. Chem.* 128, 347 (1939).



the Einstein viscosity equation, assuming the protein molecules to be spherical. This volume is then compared with that calculated by means of the Einstein-Sutherland diffusion equation for spherical molecules. Complete agreement for these volumes would mean that the protein molecules are actually spherical and the extent of disagreement is a measure of their departure from a sphere. Table 1 shows the result of these calculations, using the data of Neurath and Saum.

TABLE 1

COMPARISON OF SPHERICAL DIFFUSION VOLUME WITH SPHERICAL VISCOSITY  
VOLUME OF SERUM ALBUMIN IN UREA

Concentrated Urea, moles per liter	Ratio Diffusion Volume to Viscosity Volume
0	0.81
1.5	0.86
3.0	0.89
4.5	0.98
6.0	0.99

The results shown in Table 1 seem to indicate that serum albumin molecules become more isotropic as the urea concentration is increased. Possibly, the peptide chains approach a random coil in arrangement. Since the viscosity of serum albumin is increased by urea and since the molecules of this protein become less asymmetric, solvent must have penetrated the protein molecule and brought about swelling.

The density of the protein in solution can be directly approached by the ingenious method of varying the density of the solvent, plotting the rate of sedimentation in the ultracentrifuge against the solvent density, and extrapolating the straight line to zero rate of sedimentation; at the density of the solvent corresponding to zero rate of sedimentation, the density of the protein is equal to that of the solvent. If a volume  $v$  of anhydrous protein with density  $\rho$  becomes associated with a volume  $\Delta v$  of water with density  $\rho_w$  to form a hydrated particle of density  $\rho_s$ , the total volume of the hydrated protein particle will be  $(v + \Delta v)$  and its mass will be  $(v\rho + \Delta v\rho_w)$ . The hydrated density will then be  $(v + \Delta v)/(v\rho + \Delta v\rho_w)$ . From these results  $\Delta v$  may be readily calculated. This method was first used by Smadel, Pickels, and Shedlovsky<sup>11</sup> in their study of vaccinia; they employed as solvents solutions of salts and of urea of varying densities. Owing to the osmotic effects of these small molecules, the hydration of the protein was probably seriously changed. Later Sharp et al.<sup>12</sup> used solutions of serum albumin whose molecular weight is about 70,000 as the solvent for influ-

<sup>11</sup> J. E. Smadel, E. G. Pickels, and T. Shedlovsky, *J. Exptl. Med.* 68, 607 (1938).

<sup>12</sup> D. G. Sharp et al., *J. Biol. Chem.* 159, 29 (1945).

enza virus. Owing to the high molecular weight of serum albumin, its osmotic effects should be minor and the estimated density of the virus in solution should be close to its calculated value. These studies have been extended,<sup>13</sup> with the conclusion that some of the strains of influenza virus have as much as 2 grams of water per gram of dry virus associated with them. Evidently, molecules of this type swell and water penetrates the internal structure of the molecule.

Schachman and Lauffer<sup>14</sup> have made similar studies on tobacco mosaic virus, both in solutions of sucrose and in solutions of bovine serum albumin. In sucrose they found an apparent density of 1.27, and in serum albumin solutions they found a density of 1.13 for the virus protein. It appears possible that the lower density formed with serum albumin arises from an interaction of the serum albumin with the virus protein, the effective volume of the virus particles thus being increased; water would be immobilized between the adsorbed serum albumin molecules, with resultant lower density. The density of 1.27 found in sucrose corresponds to a hydration of about 0.37 cc. of water of hydration per cubic centimeter of dry virus. These workers<sup>15</sup> also point out that hydration can lead to significant error in the calculation of the molecular weight of proteins from sedimentation data.

### MOLECULAR-WEIGHT AVERAGES

We can average numbers in various ways. The ordinary way is to add all the numbers and divide by the number of samples; this gives the mean value. Other averages can be more meaningful. For example, an appropriate measure of the deviation from a mean is not the sum of the differences from the mean divided by the number of observations but rather the standard deviation, which is the square root of the sum of the square of the differences divided by the number of observations.

The osmotic pressure exerted by a given number of small molecules is the same as the osmotic pressure exerted by a like number of large molecules, and, accordingly, since the osmotic pressure is proportional only to the number of units in solution, we arrive at a number-average molecular weight. In light scattering, the larger particles will scatter more light than the smaller particles and so the total scattering is not proportional to the total number of particles alone but likewise involves the weight of the particles. The calculated molecular weight is known as a weight-average molecular weight.

The number-average molecular weight is obtained from osmotic pressure measurement, from spread monolayers, and from so-called end-group titra-

<sup>13</sup> D. G. Sharp, D. Beard, and J. W. Beard, *J. Biol. Chem.* **182**, 279 (1950).

<sup>14</sup> H. K. Schachman and M. A. Lauffer, *J. Am. Chem. Soc.* **71**, 536 (1949).

<sup>15</sup> H. K. Schachman and M. A. Lauffer, *J. Am. Chem. Soc.* **72**, 4266 (1950).

ions. This last method is a chemical one in which the total number of molecules present is determined by a chemical analysis of some functional group in the molecule. The number-average molecular weight is given by

$$M_n = \frac{\sum M_i N_i}{\sum N_i} \quad (14)$$

The weight-average molecular weight is given by such methods as light scattering and rate-ultracentrifugation and is calculated by

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} = \frac{\sum C_i M_i}{\sum C_i} \quad (15)$$

Still another average, the  $z$ -average molecular weight, is obtained from the ultracentrifuge by the equilibrium method. The  $z$ -average molecular weight is

$$M_z = \frac{\sum N_i M_i^3}{\sum N_i M_i^2} = \frac{\sum C_i M_i^2}{\sum C_i M_i} \quad (16)$$

It is evident that, for a completely homogeneous, monodispersed substance, all these averages are equal to each other  $M_n = M_w = M_z$ . It is only for a heterogeneous dispersion of molecular sizes that the averages differ from each other.

To make these relations somewhat clearer, suppose we have a sample of a high-molecular-weight substance whose composition corresponded to the following:

Number Moles	Molecular Weight
5	5,000
10	10,000
15	15,000
15	20,000
10	25,000
5	30,000

The number-average molecular weight of this mixture is calculated to be 17,500, whereas the weight-average is 20,238. In any heterogeneous mixture of sizes the weight-average molecular weight is always larger than that of the number-average molecular weight. In fact, the ratio of these two averages is a rough measure of the degree of heterogeneity.

As we have noted, the molecular weight can be calculated from osmotic-pressure measurements, from force-area measurements on spread monolayers, from light scatter, and from equilibrium centrifugation without any other additional information. In order to obtain the molecular weight from rate sedimentation, one must also perform a diffusion measurement. It is not, in general, possible to obtain the molecular weight of a high biopolymer



from viscosity measurements alone. However, Neurath, Cooper, and Erickson<sup>16</sup> were able to arrive at an approximate value of the molecular weight of proteins by combining viscosity and diffusion studies. They estimated the asymmetry of the protein molecules from viscosity measurements, using Simha's equation, and from this asymmetry they calculated the ratio of  $D_0/D$  by means of the Herzog, Llig, and Kadar diffusion equation. Knowing the value of  $D$  from experiment they were then able to estimate  $D_0$ , and, accordingly, the molecular weight could be calculated. Such methods are useful if more direct methods are not available. All the methods that we have considered give anhydrous molecular weights because the concentration is always expressed on an anhydrous basis.

In principle, the asymmetry of the molecule can be obtained from viscosity, from diffusion along with a knowledge of the molecular weight, from measurements on the rotary diffusion constant, and from rate sedimentation and the diffusion constant. Such measurements, however, suffer from a number of ambiguities. One of these is hydration; another is the specific shape of the molecule, i.e., whether the molecule is a random coil, a prolate ellipsoid of revolution, a regular figure, such as a prism with edges, etc.

If the molecules are very elongated, it is to be expected that such calculations will be more successful than if the molecules approach more nearly a symmetrical figure. For example, using a volume hydration of 15 per cent, Schachman and Lauffer<sup>17</sup> calculate from viscosity an axial ratio 18.3 for tobacco mosaic virus, and from diffusion and sedimentation a ratio 17.9. Direct measurements with an electron microscope<sup>18</sup> give an axial ratio 23.0. The agreement between these results can be considered excellent.

## PROBLEMS AND QUESTIONS

1. Define partial specific volume of a solute, and describe briefly how to measure it.
2. Calculate the number-average and weight-average molecular weights of the high-molecular-weight substance whose molecular-weight distribution is given on page 324 and satisfy yourself that the values in the text are correct.
3. The diffusion constant of a certain protein was measured at 25° C. and found to be  $8.86 \times 10^{-7}$  sq. cm. per second. The volume intrinsic viscosity based on the partial specific volume of the protein was calculated to be 5.13. Estimate the approximate molecular weight of this protein on the assumption that the molecule is a prolate ellipsoid of revolution.
4. List all the methods that have been considered for the determination of the molecular weight of high-molecular-weight substances. Classify these methods as to whether they yield number averages or weight averages.
5. List the various methods for the estimation of asymmetry of high-molecular-weight molecules, and describe the influence of hydration on each method.

<sup>16</sup> H. Neurath, G. R. Cooper, and J. O. Erickson, *J. Biol. Chem.* 138, 411 (1941).

<sup>17</sup> M. A. Lauffer, *J. Am. Chem. Soc.* 66, 1188 (1944).

## Chapter

# 16

## HIGH POLYMERIC STRUCTURES

Structure and organization are of paramount importance to biology; the "biological molecule" is the living cell. When the contents of such cells are extracted by various agents, however mild, the unique structure of the cell is destroyed and the study of materials so extracted yields limited information concerning their original function. The tools available at the present time for the investigation of the organization of intact cells are extremely limited. In this chapter, it is proposed to review some of these instruments for the investigation of cell structure, as well as structures formed by the in vitro combination of high biopolymers.

### GELS

Gels are characterized by rigidity or a structure containing a relatively small amount of solute in a large amount of solvent. An excellent review of protein gels has been written by Ferry.<sup>1</sup>

The classic example of a gel is presented by gelatin in water. Such a gel is typically formed by dissolving the gelatin in hot water and allowing the solution to cool. There are, however, many examples of gels such as fibrin clots, vitreous humor, Wharton's jelly, and protoplasm itself, as well as many others.

It is evident that, in order for a gel to form, there has to be a marked interaction between the particles present. Numerous theories of gel structure have been advanced, such as the "brush-heap" entanglement of highly elongated particles, the solvation theory of large particles, and the formation of network structure which extends throughout the volume, immobilizing the entrapped liquid. All these theories have one point in common; all stress the importance of large particles or molecules.

The formation of a proper gel with a degree of rigidity is undoubtedly due to the growth of a three-dimensional network throughout the volume of the solution.

<sup>1</sup> J. D. Ferry, *Advances in Protein Chem.* 4, 1 (1948), edited by M. L. Anson and J. T. Edsall, Academic Press, Inc., New York.

Flory<sup>2</sup> has proposed a theory of gelation and has defined certain conditions that are necessary for gelation. He points out that polymerizations which are propagated through the intermolecular reactions of "bivalent" or bifunctional molecules lead to soluble, diffusible products, whereas the incorporation of units of higher functionality permits formation of gelled or insoluble products.

Consider, as Flory has done, a substance containing groups *A* and *B*. *A—A* and *B—B* represent bifunctional units, whereas  $A-\begin{array}{c} \diagup A \\ \diagdown A \end{array}$  represents the trifunctional branch unit. *AB* or *BA* is the product of the condensation of two functional groups. We then have the situation pictured in Fig. 1.

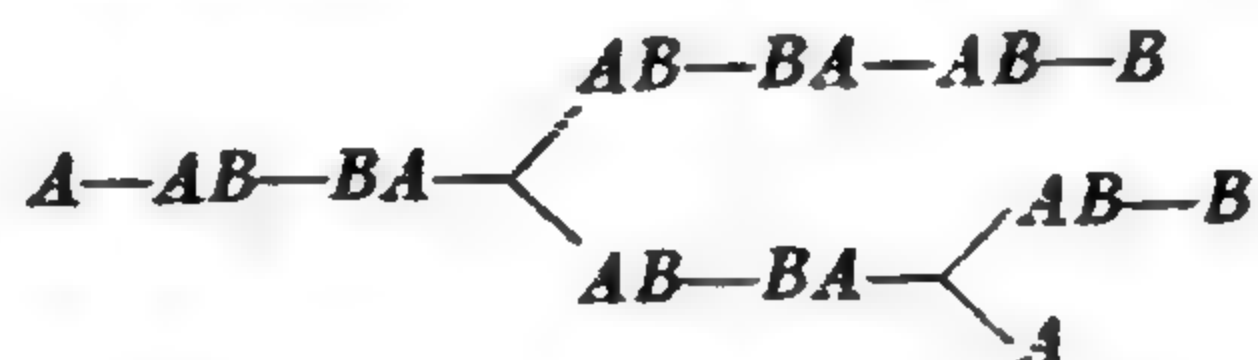


FIG. 1. Trifunctionally branched polymers. (Flory.)

Let  $\alpha$  be the probability that a chain leading from a trifunctional unit eventually leads to another branched unit. If  $\alpha < \frac{1}{2}$ , the indefinite extension of a network is impossible since a given chain has less than an even chance of reproducing two new chains. On the other hand, if  $\alpha > \frac{1}{2}$ , branching of successive chains may continue the network indefinitely. The condition  $\alpha = \frac{1}{2}$  is, therefore, critical for the formation of infinite structures or for the formation of a gel. Flory gives the equation for the general case, which turns out to be

$$\alpha_c = \frac{1}{(f - 1)} \quad (1)$$

where *f* is the functionality of the branching units interpolymerized at random with bifunctional units.  $\alpha_c$  is the critical probability above which a gel may form and below which no gel can form. As proteins, in general, contain many reactive groups, the possibility of gel formation is large.

Ordinarily when a solution changes to a gel there is little change in the gross physical and chemical properties of the system aside from the obvious appearance of mechanical structure. The electrical conductance through a gel in the presence of electrolytes is substantially the same as it is in the liquid condition. On the other hand, if the electrolyte content of a gelatin solution is quite small, the solution has a slightly higher con-

<sup>2</sup> P. J. Flory, *J. Phys. Chem.* 46, 132 (1942).



ductance than the gel.<sup>3</sup> Freundlich and Abramson<sup>4</sup> found that the electrophoretic mobility of quartz particles is the same through a gelatin gel as through the gelatin solution. They believed there was a local liquefaction of the gel in the immediate neighborhood of the particles which permitted their unhindered movement through the gel.

Freundlich,<sup>5</sup> on the basis of Heyman's<sup>6</sup> studies on the volume changes on gelation, was able to classify gels into three classes. Thixotropic gels show no volume change when gelation or solution occurs at a fixed temperature. The second type, exemplified by gelatin or agar, displays a small decrease of volume with setting; heat is evolved in the process. The third type which is represented by aqueous solutions of methylcellulose shows a small increase of volume and an absorption of heat during gelation. Thus, it is found that gels of the second type tend to set upon cooling, whereas those of the third type liquefy as the temperature falls. Heller<sup>7</sup> has studied sol-gel transformations in mixtures of gelatin and of methylcellulose dissolved in water. Upon standing such a mixture separates into two phases. The lower phase is largely gelatin, and the upper is largely methylcellulose. Upon cooling such a separated system, the lower phase sets to a gel, while the upper phase containing the methylcellulose remains liquid. Heating above 50° C. produces the opposite effect; the upper phase sets, and the lower phase remains liquid.

### THIXOTROPY

Some gels when mechanically agitated undergo a reversible, isothermal gel-sol transformation; the gel re-forms when the agitation is stopped. The word thixotropy is derived from the Greek "thixis" meaning a touch and "trope" meaning a turn, to change.

Almost any gel will show a certain degree of thixotropy, but some gels show this property to a particularly striking degree. Among these may be mentioned vanadium pentoxide, benzopurpurin, dibenzoyl-cystine, barium malonate, iron oxide, and certain clays such as bentonite. The dispersion medium for all these substances is water. The concentration of the suspended material is important. If it is too concentrated, the gel cannot be liquefied by shaking; if it is too dilute, no gel will form. The electrolyte concentration is also critical.

The clay bentonite is very convenient for the demonstration of thixotropy. The particles are very asymmetric, being long, thin plates. Ben-

<sup>3</sup> D. M. Greenberg and M. A. Mackey, *J. Gen. Physiol.* 15, 161 (1931).

<sup>4</sup> H. Freundlich and H. A. Abramson, *Z. physik. Chem.* 133, 51 (1928); *J. Gen. Physiol.*, 11, 743 (1928).

<sup>5</sup> H. Freundlich, *J. Phys. Chem.* 41, 901 (1937).

<sup>6</sup> E. Heyman, *Trans. Faraday Soc.* 31, 846 (1935); 32, 462 (1936).

<sup>7</sup> W. Heller, *J. Phys. Chem.* 45, 378 (1941).

tonite is derived from volcanic dust, and its main constituent is the mineral montmorillonite. It is one of the few inorganic substances that swell in water. To obtain a thixotropic gel of bentonite, water is mixed with the clay until the desired consistency is obtained. The amount of water added determines the setting time of the gel. If the clay suspension is quite concentrated, it is possible to hear the movement of the liquefied suspension in response to a vigorous shaking of the gel in a test tube; but the gelling time may be so short that as soon as the shaking has stopped the gel sets and the liquid state is not observed.

Electrolytes added to the proper sol will produce a thixotropic system, the effectiveness of electrolytes in regard to valence, etc., exactly paralleling the influence of electrolytes on coagulation of sols. Thixotropy may be regarded in a sense as the first step in the direction of coagulation. Increasing temperature decreases the time required for a thixotropic sol to set to a gel just as increasing temperature increases the rate of coagulation of a sol.

There is a marked correlation between the asymmetry of particles and their tendency toward thixotropy. The more asymmetric the particles, the more likely the substance is to exhibit marked thixotropy.

The formulation of a theory of thixotropy has occupied the attention of a number of workers.<sup>8</sup> One necessity for understanding thixotropy is the conception of long-range forces. These forces must be strong enough to hold the particles in place, but at the same time they cannot be so strong as to prevent rupture upon the application of comparatively weak mechanical forces. They must also be of a non-specific character; otherwise the speed of interaction between particles of such size would be very slow. The nature of these forces is primarily electrostatic, but it is incompletely understood. They have been discussed to some extent in Chapters 9 and 13.

## SWELLING OF GELS

A characteristic feature of dry proteins is their tendency to imbibe water and to swell. Dry isoelectric gelatin when placed in water imbibes water and swells to a definite limit, whereas other proteins, such as egg albumin, swell indefinitely and eventually disperse completely. Proteins swell as a result of two factors: (1) The polar groups on the protein molecules are hydrated, and water is taken up through hydrogen bonds. (2) The entropy of mixing is positive just as the mixing of two similar liquids takes place with a positive entropy change; the greater randomness of the mixture makes the mixing a spontaneous process.

The intermolecular bonds between gelatin molecules prevent the solution of gelatin and confine the swelling to a definite limit. If the temperature

<sup>8</sup> H. Freundlich, *Thixotropy*, Hermann et Cie., Paris, 1935.



be increased sufficiently, the weak intermolecular bonds are broken and the gelatin disperses completely; however, the bonds reform when the temperature is lowered. The dispersion of a gel at higher temperatures is roughly analogous to the melting of a crystal, although the solution temperature is far from sharp and the solution temperature is always appreciably higher than the gelatin temperature. The melting point of a gelatin gel is very dependent on the concentration of the gelatin<sup>9</sup> and can be expressed over a portion of the concentration region by the relation  $\ln c = a - b/RT$ , where  $b$  has the dimension of a heat effect, a "molar heat of gelation."

The swelling pressure of a gel can be calculated from the lowering of the vapor pressure of the liquid associated with the gel. Consider again the relation between the vapor-pressure lowering and the osmotic pressure as discussed in Chapter 12. On the basis of the maximum work principle, we obtain

$$P_h \bar{V}_1 = RT \ln \frac{P_0}{P} \quad (2)$$

where  $P_h$  is the hydrostatic pressure of a solution whose vapor pressure is  $P$  in equilibrium with the pure solvent whose vapor pressure is  $P_0$ .  $\bar{V}_1$  is the partial molar volume of the solvent. We can use equation 2 to calculate the swelling pressure of a gel, since we can set  $P_h$  equal to the swelling pressure. The swelling pressures exerted by proteins at low moisture content are rather impressive. For example, the swelling pressures calculated by means of equation 2 at 25° C. for isoelectric salt-free gelatin are 3900 atmospheres per square centimeter for 5 grams of water per 100 grams of gelatin, 2200 atmospheres per square centimeter for 10 grams of water, and 600 atmospheres per square centimeter for 20 grams of water per 100 grams of gelatin.

It will be recognized that the term  $P_h \bar{V}_1$  in equation 2 is equal to the free energy change involved in the hydration of the gel. The free energy change can be resolved into a heat term and into the entropy of swelling in analogy with the resolution of the osmotic pressure into these terms, discussed in Chapter 12. This is the basis for the theoretical treatment of Flory and Rehner<sup>10</sup> regarding the relation between the maximum extent of swelling and the average molecular weight of the gel molecules between cross-links. In the case of gels of polar biopolymers, however, the interaction between the solvent and the particles is so extensive that the quantitative application of this theory hardly appears profitable.

If the pH of the completely swollen isoelectric gelatin gel is shifted either to the acid or the basic side of the isoelectric point, additional swelling is noted. The reason for the increased swelling is undoubtedly due to the

<sup>9</sup> R. S. Gordon, Jr. and J. D. Ferry, *Federation Proc.* 5, 136 (1946).

<sup>10</sup> P. I. Flory and J. Rehner, Jr., *J. Chem. Phys.* 11, 521 (1943).



electrical charges on the gelatin and to the development of a Donnan equilibrium, as originally postulated by Proctor and Wilson.<sup>11</sup> This point of view was championed by Loeb<sup>12</sup> in his well-known book. As pointed out in Chapter 12, a Donnan equilibrium leads to an increased osmotic pressure due to the accumulation of electrolytes inside the gel, this accumulation being brought about by the electrically charged groups on the protein. The gel then swells in response to the increased osmotic pressure.<sup>13</sup>

### RIGIDITY OF GELS

The modulus of rigidity or shear is a measure of the strength of gel structure. The modulus of rigidity  $G$  is defined<sup>14</sup> as

$$G = \frac{\text{Stress}}{\text{Strain}} = \frac{2FxL}{\pi r^4 \theta} \quad (3)$$

where  $x$  is the distance in centimeters from the center of a bar of gel and  $Fx$  is the torque applied which produces a twist of  $\theta$  radians in a bar of length  $L$  and of radius  $r$ .

The measurement of the modulus of rigidity involves two difficulties. One is viscosity. That is, twisting a bar of a highly viscous liquid requires the application of a torque because of the high viscosity, and yet the liquid may have no rigidity. The second difficulty involves the relaxation time. The stress required to produce a given strain decays with time (the material relaxes), and, if the measurement requires a greater time to perform than the relaxation time, little information can be derived from such measurements.

When the relaxation times are of the order of minutes or longer, a static experiment will suffice and correction for viscous displacement can be made by measuring the recovery after removal of load. Materials too weak to support their own weight can be sheared between coaxial cylinders.

When relaxation times are less than a second, however, it is necessary to use either impulsive or alternating stresses with periods shorter than the relaxation times. For various devices for subjecting gels to mechanical stresses the following references may be consulted.<sup>15, 16, 17, 18, 19, 20</sup>

<sup>11</sup> H. R. Proctor and J. A. Wilson, *J. Chem. Soc. (London)* 109, 307 (1916).

<sup>12</sup> J. Loeb, *Proteins and the Theory of Colloid Behavior*, McGraw-Hill Book Co., New York, 1922.

<sup>13</sup> D. Jordan Lloyd and W. B. Pleass, *Biochem. J.* 21, 1352 (1927).

<sup>14</sup> S. Timoshenko, *Theory of Elasticity*, McGraw-Hill Book Co., New York, 1934.

<sup>15</sup> T. Schwedoff, *J. phys.* [2] 8, 341 (1889).

<sup>16</sup> E. Hatschek and R. S. Jane, *Kolloid-Z.* 39, 300 (1926).

<sup>17</sup> J. D. Ferry and G. S. Parks, *Physics* 6, 356 (1935).

<sup>18</sup> A. P. Alexandrov and Y. S. Lazurkin, *Acta Physicochim. U.R.S.S.* 12, 647 (1940).

<sup>19</sup> J. M. Kendall, *Rheol. Bull.* 12, 26 (1941).

<sup>20</sup> W. Philippoff, *Physik. Z.* 35, 883 (1934).

Ferry<sup>22</sup> has proposed an optical method which is suitable for transparent materials. It is based on the visualization of the propagation of a strain in a gel produced by an alternating transverse stress. The strain travels through the gel as transverse waves and causes the gel to become birefringent. By the proper optical equipment it is possible to obtain the frequency and the wavelength of the transverse waves from which their velocity may be calculated. The velocity of transverse waves is

$$V = \left( \frac{G}{\rho} \right)^{1/2} \quad (4)$$

where  $\rho$  is the density of the material.

Ferry and his co-workers have applied this method to gels formed from fibrinogen<sup>22</sup> and gelatin gels.<sup>23</sup>

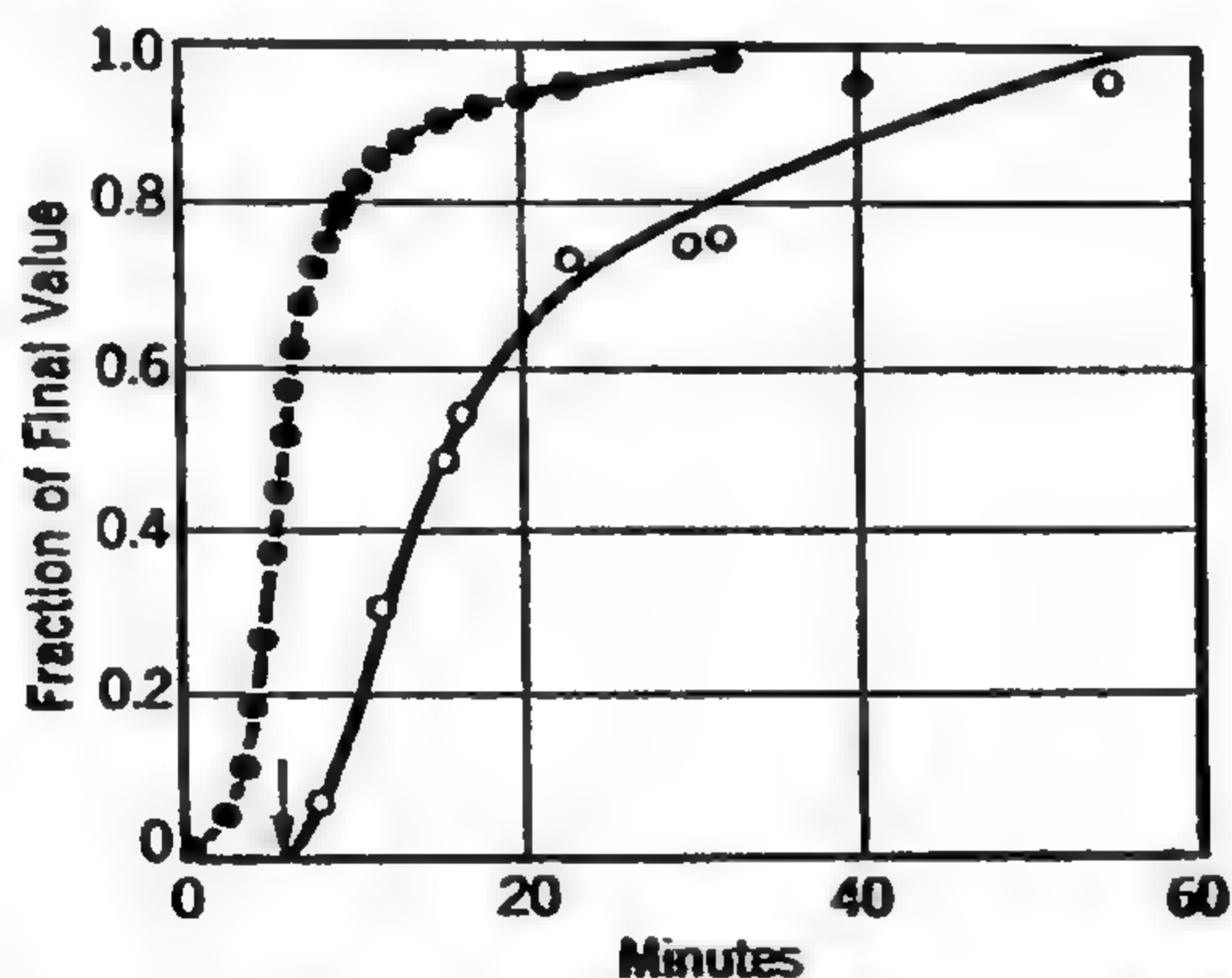


FIG. 2. Rigidity (○) and opacity increase (●), plotted against time, during clotting of solution with fibrinogen concentration 16 grams per liter, thrombin 1.0 unit per cc., ionic strength 0.3. pH 6.33. Arrow denotes clotting time. (Ferry and Morrison.)

The modulus of rigidity of gelatin gels is a straight-line function of the square of the gelatin concentration. This modulus decreases very drastically with increasing temperature. It is also very dependent on the molecular weight of the gelatin samples; increasing more or less linearly with increasing molecular weight. As the gelatin sol sets to a gel there is a sharp increase in the modulus of rigidity, followed by a slow increase as the gel ages.

The work of Ferry and Morrison<sup>22</sup> on gels or clots formed from fibrinogen is also of great interest. When thrombin is added to a solution of fibrinogen a clot is formed. The nature of this clot (opaque or transparent) is depend-

<sup>22</sup> J. D. Ferry, *Rev. Sci. Instruments* 12, 79 (1941).

<sup>22</sup> J. D. Ferry and P. R. Morrison, *J. Am. Chem. Soc.* 69, 388 (1947).

<sup>23</sup> J. D. Ferry, *J. Am. Chem. Soc.* 70, 2244 (1948).



ent on a number of factors such as pH and electrolyte concentration. The time of clotting is also a function of a number of factors. Shown in Figure 1 is a plot of the increase in opacity and rigidity of fibrinogen under the clotting.

### ELASTICITY OF FIBERS

Biological fibers, which may be regarded as linear gels, can be characterized by their modulus of stretch or of elasticity which is

$$E = \frac{\text{Stress}}{\text{Strain}} = \frac{FL}{A \Delta L} \quad (5)$$

where  $F$  is the stress in dynes,  $A$  is the cross-sectional area of the fiber,  $L$  is the length of the fiber, and  $\Delta L$  is the increase in length of the fiber produced by the stress.

Shown in Table 1 is the stretch modulus of elasticity for some diverse materials.

TABLE 1  
MODULI OF ELASTICITY IN DYNES PER SQUARE CENTIMETER

Substance	$E$
Copper wire	$11 \times 10^{11}$
Steel wire	$20 \times 10^{11}$
Rubber	
$-200^\circ \text{C.}$	$1 \times 10^{11}$
$20^\circ \text{C.}$	$8 \times 10^6$
Human hair	$3.6 \times 10^9$
Contracted striated muscle	$4 \times 10^8$
Silk	$1 \times 10^{11}$

Under stress, the modulus of elasticity decays with time according to the relation

$$E = E_0 e^{-t/\lambda} \quad (6)$$

where  $t$  is the time during which the stress is allowed to act and  $\lambda$  is the relaxation time; as is apparent from equation 6,  $\lambda$  is the time required for the modulus to decay to  $1/e^{2.3}$  part of the initial modulus. For two or more elastic elements (so-called Maxwell elements) acting in parallel the observed modulus is given by the relation

$$E = E_0' e^{-t/\lambda_1} + E_0'' e^{-t/\lambda_2} + \dots \quad (7)$$

The stretching of any elastic material involves viscosity as well as elasticity, and, accordingly, stretching of a fiber can become a complicated



process. The relation between the viscosity, the moduli of elasticity, and the relaxation times for elastic elements in parallel is given by Kuhn.<sup>24</sup>

$$\eta = \frac{1}{2(1 + \mu)} (E_1\lambda_1 + E_2\lambda_2 + \dots) \quad (8)$$

where  $\mu$  is Poisson's ratio and is defined as the ratio of the transverse contraction per unit dimension of a bar of uniform cross section to its elongation per unit length, when subjected to a tensile stress; it usually is close to 0.5.

If the relaxation time is very short, a body can exhibit a high modulus of elasticity which decays rapidly; this behavior is shown by "bouncing putty" which can be deformed by slow application of stress as can ordinary putty (a plastic body); yet if the bouncing putty is dropped on the floor it will bounce like a rubber ball. One can attain any quality of elastic behavior by variation in the modulus, the relaxation time, and the viscosity.

From a molecular point of view there are two kinds of elasticity:<sup>25</sup> (1) rubber-like and (2) normal body elasticity. In rubber-like elasticity the heat motion of the molecules is principally responsible for the elastic behavior, whereas in the second case which is shown by steel wire and most bodies the distortion of chemical bonds is responsible. Rubber-like elasticity is characterized by three conditions:

1. Heat is evolved when the fiber is stretched.
2. Fiber contracts when heated under a given stress.
3. The stress of the extended fiber increases with increasing temperature.

Wiegand and Snyder<sup>26</sup> proposed an equation based on thermodynamics which enabled them to separate the stress of an elastic body into the part due to internal energy and the part due to the heat motion (entropy change). This equation has been used by Guth and others to analyze the thermal data on rubber, by Wöhlisch,<sup>27</sup> by Meyer and Pickens,<sup>28</sup> by Wood,<sup>29</sup> and by others<sup>30</sup> on similar data from the study of the elastic properties of muscle, collagen, elastin, and keratin. According to Wiegand and Snyder the load can be resolved as follows

$$F = F_u + F_s \quad (9)$$

<sup>24</sup> W. Kuhn, *Angew. Chem.* 52, 289 (1939).

<sup>25</sup> W. B. Wiegand, *Trans. Inst. Rubber Ind.* 1, 141 (1926).

E. Guth, *Surface Chemistry*, Pub. Am. Assoc. Advanc. Sci., No. 21, p. 103, 1943.

<sup>26</sup> W. B. Wiegand and J. W. Snyder, *Trans. Inst. Rubber Ind.* 10, 141 (1934).

<sup>27</sup> E. Wöhlisch, *Arch. ges. Physiol (Pflügers)* 246, 469 (1942).

<sup>28</sup> K. H. Meyer and L. E. R. Pickens, *Proc. Roy. Soc. (London)* B124, 29 (1937).

<sup>29</sup> H. J. Wood, *J. Colloid Sci.* 1, 407 (1946).

<sup>30</sup> H. B. Bull, *J. Am. Chem. Soc.* 67, 533 (1945).

where  $F$  is the total force exerted by the elastic body,  $F_u$  is the force due to the internal energy, and  $F_s$  is the entropy term. The entropy term  $F_s$  is given by the relation

$$F_s = T \left( \frac{dF}{dT} \right)_L \quad (10)$$

Accordingly, if we plot the stress against the temperature and multiply the slope of the line by the absolute temperature, we obtain  $F_s$ . Shown in Fig. 3a is the resolution of the stress for vulcanized rubber, and in Fig. 3b is the resolution of the stress for human hair.

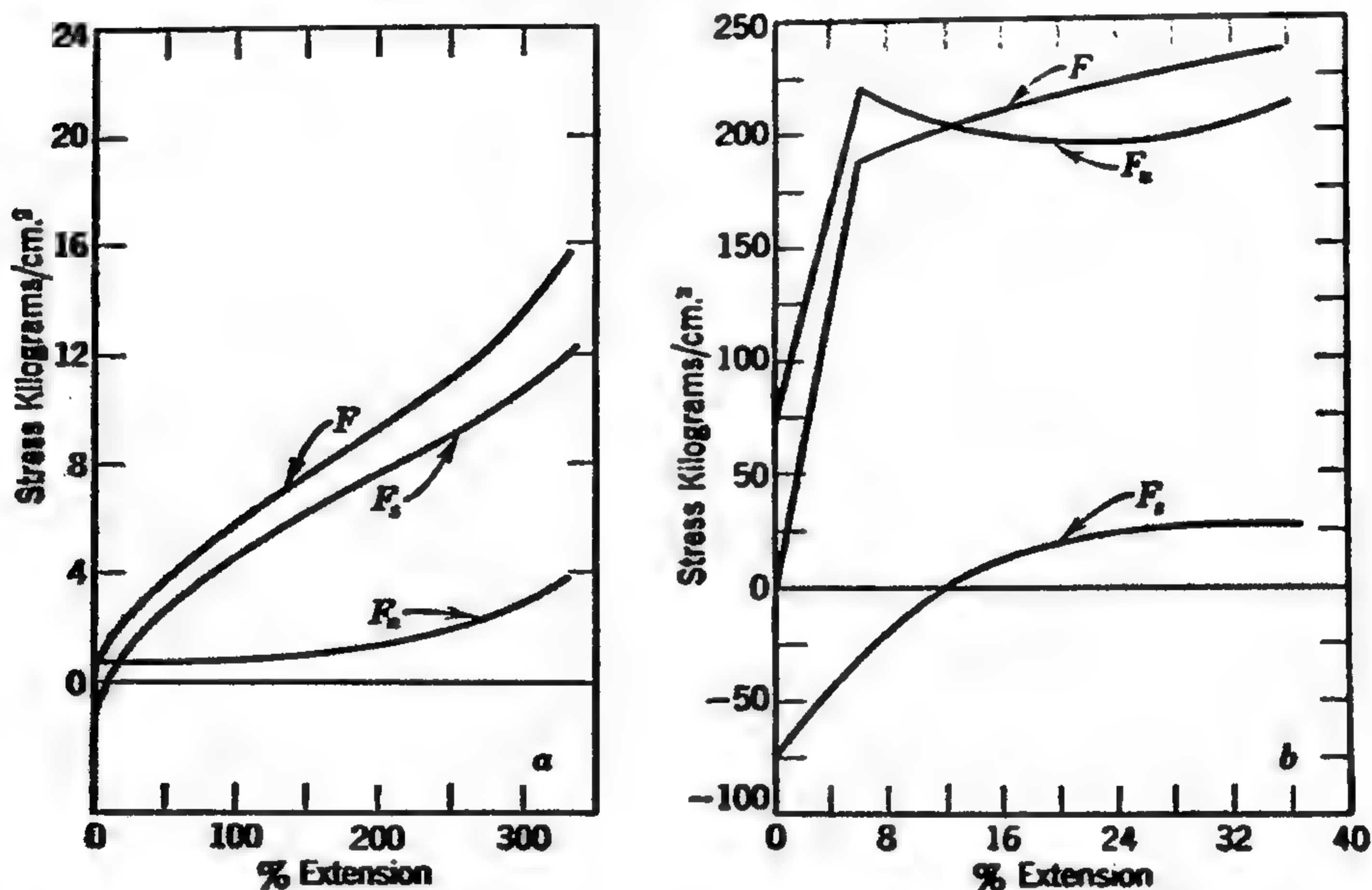


FIG. 3. Stress plotted against per cent extension at 25°C. Total stress ( $F$ ), entropy term ( $F_s$ ) and the stress due to internal energy ( $F_u$ ). (a) Rubber (Guth). (b) Human hair saturated with water.

Evidently, heat motion (Brownian motion of the molecular chains) plays a much more conspicuous role in the elastic properties of rubber than it does in the stretching of hair which contains numerous polar groups capable of interaction.

As pointed out in Chapter 11, a stretched fiber molecule in solution tends to form a random coil. It has this property because a random coil is a more probable state than is the stretched form; the coiling involves a positive entropy change. Increase in temperature increases the molecular vibration of the fiber molecules and increases the molecular forces tending to produce a coil; this is why an increase of temperature increases the stress

on a stretched rubber band. By the application of statistical mechanics it is possible to derive a relation between the stress due to entropy and the extent of stretch.<sup>31, 32, 33</sup> This relation can be expressed as

$$F_s = \alpha \left( L - \frac{1}{L^2} \right) \quad (11)$$

where  $\alpha$  is a coefficient involving the molecular weight of the segments of the fiber. We have further, upon differentiating equation 11,

$$\frac{dF_s}{dL} = E = \left( 1 + \frac{2}{L^3} \right) \alpha \quad (12)$$

Evidently, when  $L$  is unity, i.e., at zero extension,  $E$  is equal to  $3\alpha$ . For randomly kinked molecular chains connected by cross-links,  $E$  is equal to  $3RTw/M$ , where  $M$  is the average molecular weight of the fiber molecules between cross-links,  $w$  is the weight of the elastic material per cubic centimeter,  $R$  is the gas constant, and  $T$  is the absolute temperature. Kuhn<sup>34</sup> has considered randomly oriented rigid rods connected end to end by flexible links and imbedded in an isotropic fluid matrix. The modulus of elasticity at zero extension that he obtained for such a system is  $9RTw/5M$ .

Let us consider the entropy involved in muscle contraction.<sup>35</sup> Basically, there are two ways in which a muscle can contract. The contraction is due to a change in internal energy, or the muscle is a heat engine and the entropy change is responsible for the contraction. In the first case, the elasticity resembles that of a normal body, whereas in the second case the elasticity is rubber-like in nature. If the contraction of a muscle is principally due to an increase in entropy, the contraction of a muscle is spontaneous and the relaxation of the muscle requires the expenditure of energy on the part of the muscle.

An immediate difficulty is encountered when a comparison between rubber and muscle is attempted. The resting state of muscle is its extended state, whereas the resting state of rubber is its contracted state. When a muscle is allowed to contract isometrically (contraction at a fixed length), the tension exerted becomes progressively less as the length of the muscle is made smaller relative to its resting length. The plot of the isometric tension of a muscle fiber as a function of the length of the contracted fiber is clearly a stress-strain curve of the muscle. The problem is then to ex-

<sup>31</sup> E. Guth and H. M. James, *Ind. Eng. Chem.* **33**, 624 (1941).

<sup>32</sup> P. J. Flory and J. Rehner, Jr., *Ann. N. Y. Acad. Sci.* **44**, 419 (1943).

<sup>33</sup> F. T. Wall, *J. Chem. Phys.* **10**, 132, 485 (1942).

<sup>34</sup> W. Kuhn, *Kolloid-Z.* **87**, 3 (1939).

<sup>35</sup> H. B. Bull, *Quart. Bull. Northwestern Univ. Med. School* **20**, 1 (1946); *J. Am. Chem. Soc.* **67**, 2047 (1945).



trapolate this stress-strain curve to zero stress from which the length of the completely contracted fiber can be estimated. The excellent experiments of Ramsey and Street<sup>36</sup> allow such an extrapolation to be made. These workers have measured the isometric tensions exerted by single muscle fibers from the semitendinosus muscles of the frog (*Rana pipiens*).

Assigning the value of unity to the length of a completely contracted fiber, the length of the fiber at tensions greater than zero have been expressed relative to this completely contracted length. The tensions have been expressed in terms of dynes per square centimeter of cross section of the completely contracted fiber. These tensions are plotted against the relative length of the fiber. The results are shown in Fig. 4.

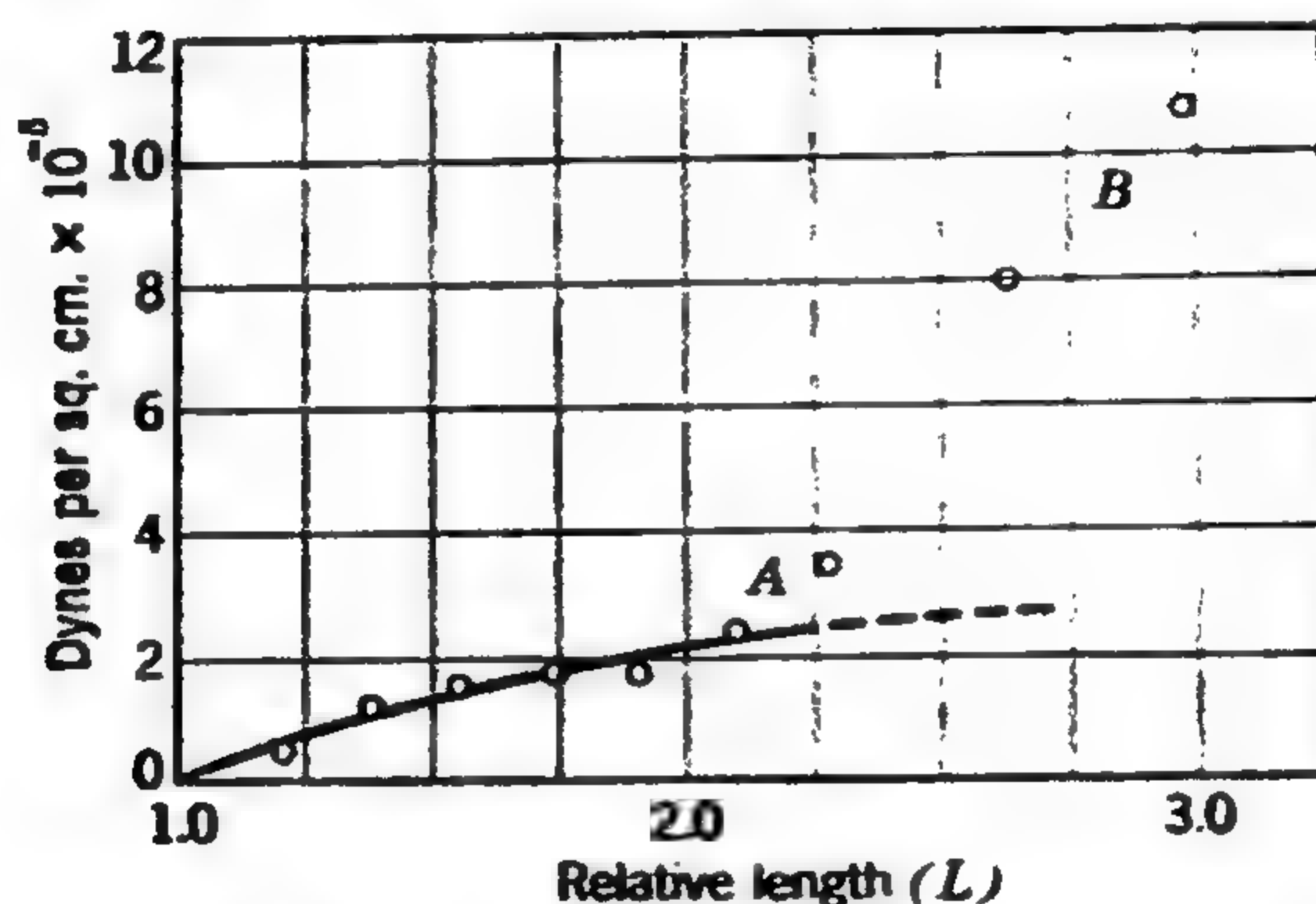


FIG. 4. Isometric tensions plotted against the relative extension of the completely contracted fiber. The points represent recalculated data of Ramsey and Street. The solid line is a plot of equation 11.

Comparison of Fig. 4 with the stress-strain curves for vulcanized rubber shows a striking similarity between the stress-strain character of rubber and that of muscle. According to statistical theory, the region from the origin to A represents the straightening of randomly kinked molecular chains of the polymer and the region from A to B represents, for the most part, a crystallization of the polymer. The modulus of elasticity of the muscle fiber at zero extension obtained from Fig. 4 is  $3.6 \times 10^5$  dynes per square centimeter.

It is generally agreed that the protein myosin is responsible for the active elastic properties of muscle. Myosin makes up about 20 per cent of the weight of the contractile element of muscle, and, accordingly,  $w$  is about 0.2. Ramsey and Street worked at  $11^\circ \text{C.}$ , and  $R$  is  $8.37 \times 10^7$  ergs per degree per mole. Using the estimated value for the modulus of elasticity noted above, we find that for randomly kinked flexible chains the molec-

<sup>36</sup> R. W. Ramsey and S. Street, *J. Cellular Comp. Physiol.* 15, 11 (1940).

ular weight of each chain is about 40,000 and for randomly arranged rigid rods the molecular weight of each rod is about 24,000.

Riseman and Kirkwood<sup>37</sup> have considered the influence of electrical charges on the myosin molecules. They suggest that the essential alteration of the structural unit, leading to a change in elastic modulus, is a change in its net electric charge. According to this view, the relaxed state of muscle is an electrically charged state and the contracted state is one in which the polypeptide chains are in an uncharged or "isoelectric" condition. These authors are inclined to the view that phosphorylation of the hydroxy amino acid residues by adenosine triphosphate provides the charging mechanism. The phosphorylation process would impart to the neutral sites originally occupied by aliphatic hydroxyl groups approximately one unit of negative charge. Subsequent dephosphorylation would remove the negative charge, the stored free energy thus being released; the muscle contracts owing to heat motion.

The increment in elastic modulus produced by the charge increment  $ne$  is

$$\Delta E = - \frac{8 N \rho n^2 e^2}{3 M DL} \quad (13)$$

where  $e$  is the elementary charge,  $n$  is the number of charges per mole,  $D$  is the dielectric constant,  $\rho$  is the density of the elastic structure, and  $L$  is the average distance between points of attachment of the elastic molecules. Substituting approximate values in equation 13, the authors calculate that the observed change in the elastic modulus from the resting to the contracting state of muscle requires that phosphorylation sites be situated about every 100 Å along the chain.

The above discussion of the elasticity of muscle is of a speculative nature; the true mechanism of muscle contraction is, as yet, unknown.

## X-RAY

One of the most useful techniques for the study of structure is that of X-ray diffraction analysis. Unfortunately, X-ray technique is rather involved both theoretically and experimentally, and all we can hope to do in this brief section is to outline some of the problems associated with the application of X-rays.

When a metal target is bombarded with high-speed electrons, X-rays are emitted. The wavelength of these rays is determined by the position of the activated electrons in the metal atom. There are  $K$ ,  $L$ ,  $M$  rays corresponding to the  $K$ -,  $L$ -,  $M$ -shells of electrons around the nucleus of the atom.  $K$ -radiation has the shortest wavelength and is more penetrating. There

<sup>37</sup> I. Riseman and J. G. Kirkwood, *J. Am. Chem. Soc.* 70, 2820 (1948).



are several electrons in each shell, and, accordingly, the rays arising from a given shell will be multiple. For example, the  $K$  X-rays are divided into  $K_{\alpha_1}$ ,  $K_{\alpha_2}$ ,  $K_{\beta_1}$ , and  $K_{\beta_2}$ . The metal targets most frequently employed are copper, iron, and chromium. Table 2 gives the wavelengths of the  $K$ -radiation of these metals.

TABLE 2

WAVELENGTHS OF  $K$  X-RAYS

Element	Wavelengths ( $\text{\AA}$ )			
	$K_{\alpha_1}$	$K_{\alpha_2}$	$K_{\beta_1}$	$K_{\beta_2}$
Chromium	2.2889	2.2850	2.0806	.....
Iron	1.9310	1.9321	1.7530	.....
Copper	1.5412	1.5374	1.3894	1.3782

It is highly desirable to have monochromatic X-rays for diffraction analysis, and the usual method for obtaining such radiation is either to absorb out the weaker rays with metal foil or to pass the X-ray beam through a large crystal and isolate the desired wavelength by diffraction.

When an X-ray strikes an object that is made up of repeating units whose distances of separation are commensurate with the wavelength of the X-ray, we obtain diffraction of the X-rays. This diffraction is exactly analogous to that observed with ordinary light passed through a diffraction grating. Diffraction involves the reinforcement of rays whose waves are in phase and the cancellation of rays whose waves are out of phase. Consider Fig. 5.

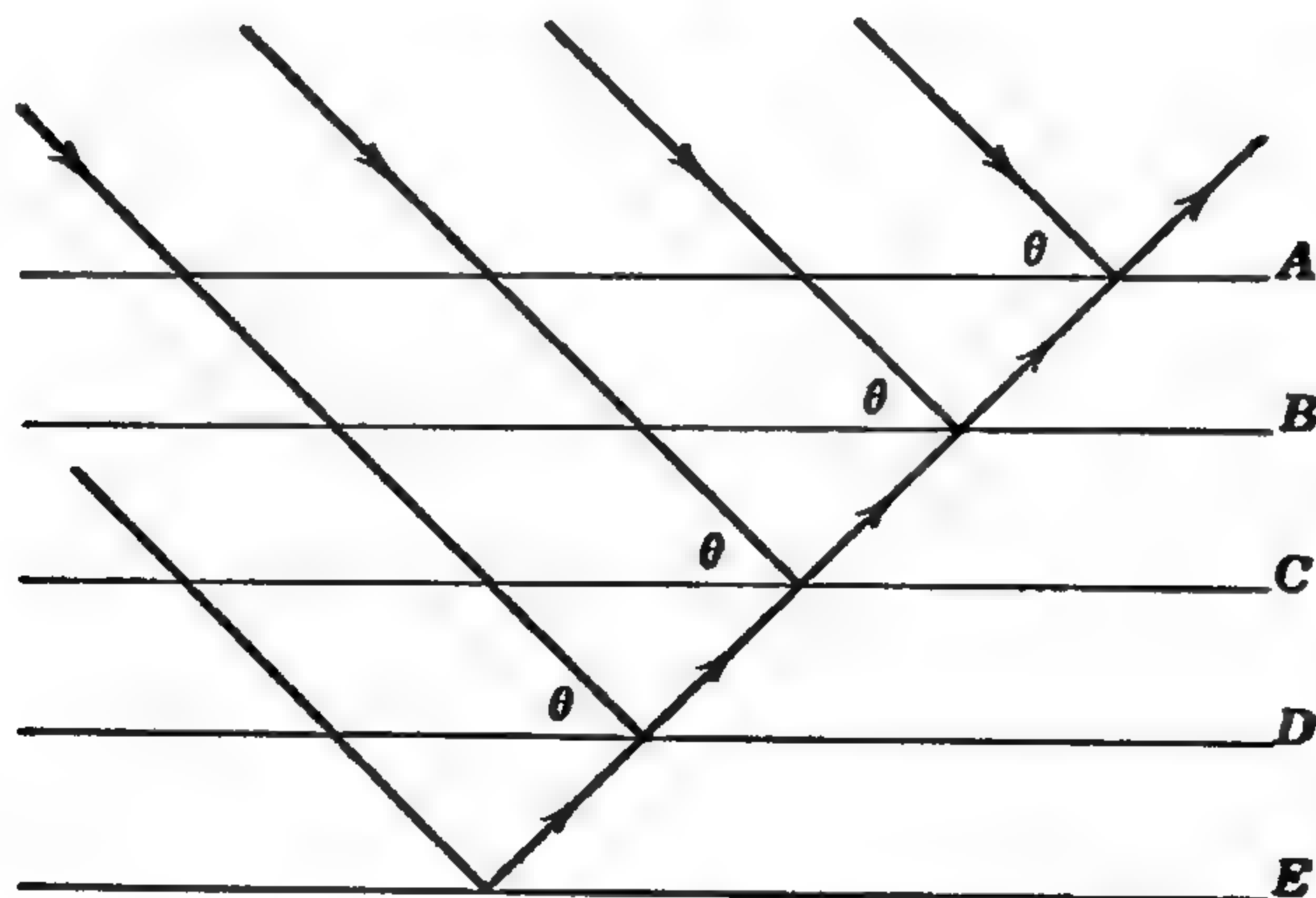


FIG. 5. X-ray reflection from equidistant planes.

A parallel beam of X-rays impinges on the crystal so that the glancing angle is  $\theta$ . Part of the beam will be reflected from plane  $A$ . Some of the beam will penetrate the crystal and be reflected from plane  $B$ , from plane  $C$ , etc. If the total distances traveled by these reflected rays differ from



each other by multiples of whole numbers of wavelengths, then the various reflections will reinforce each other and a strong diffracted beam will be observed at this particular angle of reflection.

The condition for a maximum reflection is given by Bragg's equation

$$n\lambda = 2d \sin \theta \quad (14)$$

where  $d$  is the distance between the reflecting planes. As  $\theta$  is increased a series of positions is found, corresponding to  $n = 1, 2, 3, 4$ , etc., at which maximum reflection occurs. These diffraction maxima are called first-, second-, third-, etc., order according to the value of  $n$ .

In practice, a collimated beam of monochromatic X-rays is allowed to impinge on the specimen. At a suitable distance from the specimen and in line with the undeviated beam is placed a photographic plate, and the extent of diffraction is recorded on the plate. Aside from the quantitative considerations, much can be learned from a study of the quality of an X-ray picture and the following aspects of such a picture can be listed:

1. No diffraction visible in the picture means complete disorder. This is rarely if ever seen.
2. Diffuse concentric rings indicate repeating units but no orientation present. This type of picture is given by liquids and unoriented fibers.
3. Sharp well-defined concentric rings mean that there is no preferred orientation present. However, there are repeating distances present, and these distances exhibit little deviations from mean values.
4. Concentric rings are not complete but exist rather as arcs. This is the typical fiber photograph, the angle subtended by the arcs gives an idea of the goodness of orientation, and the sharpness of the arcs gives an idea of the constancy of the repeating distance.
5. Sharp spots are given by true crystals with order in three dimensions.

As can be seen from equation 14, the distance of the spots or rings from the central beam gives an inverse measure of the length of the repeating distance.

X-ray diffraction studies have been made on numerous biological compounds, and in many instances the molecular structures of these materials have been assigned with confidence. One of the limitations of the X-ray diffraction method is that it is not, in general, possible to arrive at the unique molecular structure by X-ray studies alone; such studies must be generously supplemented by chemical and optical data.

One of the earliest X-ray diffraction investigations was that of Sponsler<sup>38</sup> and of Sponsler and Dore<sup>39</sup> on the structure of cellulose. As a result of

<sup>38</sup> O. L. Sponsler, *J. Gen. Physiol.* 9, 677 (1926).

<sup>39</sup> O. L. Sponsler and W. H. Dore, *Colloid Symposium Monograph* 4, 174 (1926).

these studies Sponsler and Dore put forward the modern concept that the glucose residues form chains that are arranged parallel to the fiber axis. The unit cell, which is the smallest volume of the crystal that contains the three-dimensional pattern of the crystal, was determined by Meyer and Misch.<sup>40</sup>

Crystals of fatty acids are especially suited to investigation by X-ray diffraction. Among others, papers by Piper<sup>41</sup> and by Francis, Piper, and Malkin<sup>42</sup> may be consulted.

Fatty acid crystals show three principal spacings. One is long and dependent on the number of carbon atoms; the other two are small, almost equal, and nearly independent of the carbon number. The long spacings correspond to approximately two molecular lengths of fatty acid, and the other two spacings correspond to the thickness of the paraffin chains. In a crystal, the carboxyl groups form the main reflecting planes and are packed in double layers alternating with double layers of methyl groups; the double layers of methyl groups do not reflect. The individual hydrocarbon chains have parallel orientation. The cross-sectional area of the carboxyl groups is 20.4 sq. Å, whereas that of the hydrocarbon chains is 18.4 sq. Å. This difference in area requires the paraffin chains to be tilted away from the normal to the crystal planes to achieve tightness of packing. Depending on the angle of tilt, fatty acids exhibit a variety of crystal forms. The tilting of the hydrocarbon chains also enables us to understand the well-known alternations in melting points existing between the fatty acids with an even and those with an odd number of carbon atoms. Owing to the tilt of the hydrocarbon chains, the distance per CH<sub>2</sub>-group between the crystal planes is greater in the odd series than in the even with a consequent greater density of packing for the even series and, accordingly, higher melting points. If the paraffin chains were oriented normal to the plane of the crystal, there should be no alternations in the melting point of fatty acid crystals.

Some of the earliest studies on proteins were made by Katz, who investigated collagen and gelatin as a function of the water content of proteins. He was able to show that one of the spacings was very dependent on the water content, whereas the other spacings were largely independent of the water content. Katz and Derksen<sup>43</sup> identified the water-dependent spacing as the distance between the peptide chains.

The work of Meyer and Mark<sup>44</sup> on silk fibroin was also a pioneer study

<sup>40</sup> K. H. Meyer and L. Misch, *Helv. Chim. Acta* 20, 232 (1937).

<sup>41</sup> S. H. Piper, *Trans. Faraday Soc.* 25, 348 (1929).

<sup>42</sup> F. Francis, S. H. Piper, and T. Malkin, *Proc. Roy. Soc. (London)* A128, 214 (1930).

<sup>43</sup> J. R. Katz and J. C. Derksen, *Rec. trav. Chim.* 51, 513 (1932).

<sup>44</sup> K. H. Meyer and H. Mark, *Ber.* 61, 1932 (1928).



and was the first to interpret the X-ray diffraction pattern in terms of a stretched peptide chain. They identified a repeating distance along the fiber axis of  $7.0 \text{ \AA}$  which is the distance occupied by two amino acid residues. The side-chain spacing was  $5.2 \text{ \AA}$ , and the "backbone" of the peptide was  $4.6 \text{ \AA}$ .

Astbury and Street<sup>45</sup> were able to show that there is a definite relation between the molecular structure and the extent of stretching of a protein fiber. Thus ordinary hair or wool exists as  $\alpha$ -keratin with a repeating distance along the fiber axis of  $5.15 \text{ \AA}$ . When hair is extended sufficiently, it is changed into  $\beta$ -keratin with a repeating distance along the fiber of  $3.32 \text{ \AA}$ , a backbone spacing of  $4.6 \text{ \AA}$ , and a side-chain spacing of  $9.8 \text{ \AA}$ . The structure of  $\beta$ -keratin is that of a stretched peptide chain. The structure of  $\alpha$ -keratin is still obscure. See, for example, papers by Ambrose and Hanby<sup>46</sup> and also by Astbury.<sup>47</sup>

Basic to the understanding of these more complex structures is the work of Corey<sup>48</sup> on the crystal structure of amino acids and peptides. Shown in Fig. 6 is a diagrammatic representation of a fully extended polypeptide chain based on the bond distances and angles assigned by Corey.

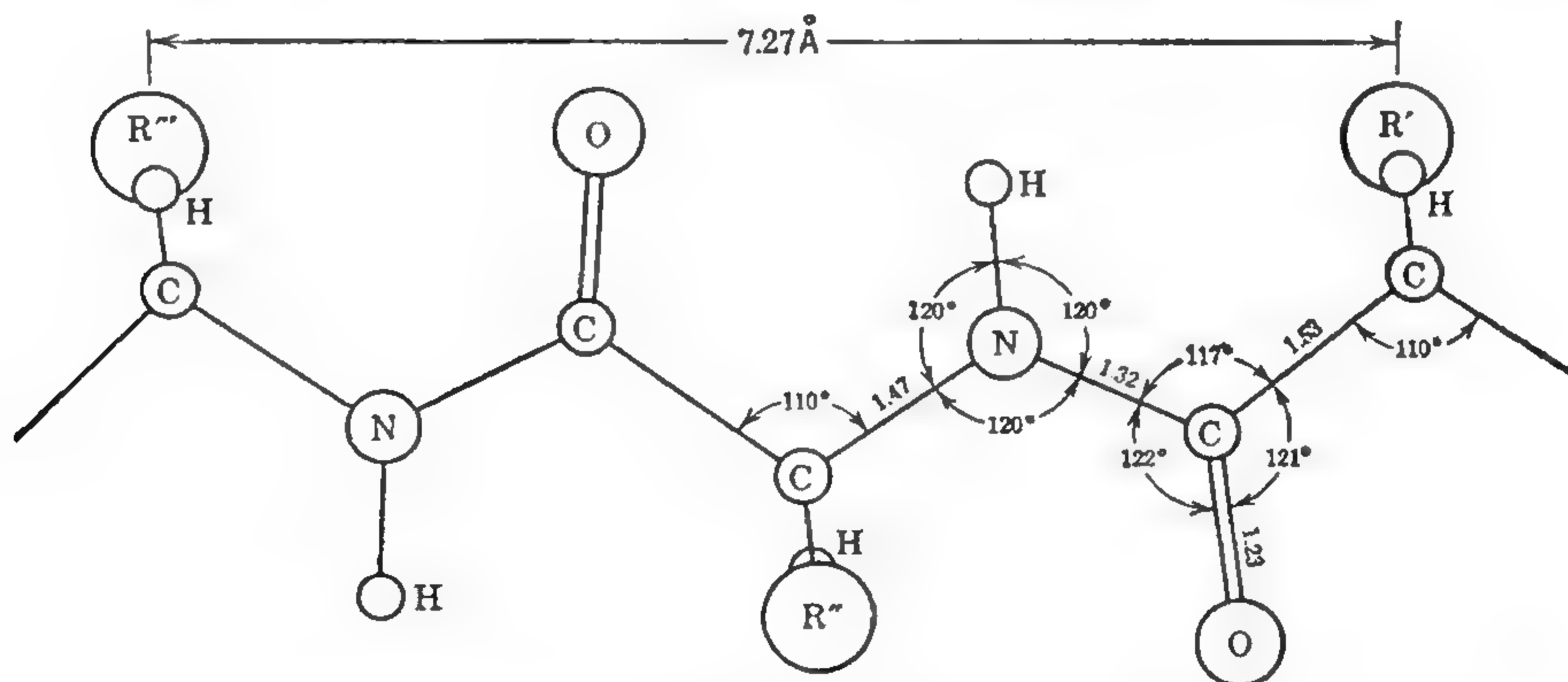


FIG. 6. Diagrammatic representation of a fully extended polypeptide chain based on bond distances and angles of Corey and Donohue.

Numerous diffraction studies have been made on crystalline proteins. Such proteins yield pictures rich in detail and definition. However, the interpretation of such photographs in terms of ultimate structure is very difficult. Use is made of the Patterson-Harker projections which involve

<sup>45</sup> W. T. Astbury and A. Street, *Trans. Roy. Soc. (London)* A230, 75 (1931).

<sup>46</sup> E. J. Ambrose and W. E. Hanby, *Nature* 163, 483 (1949).

<sup>47</sup> W. T. Astbury, *Nature* 164, 439 (1949).

<sup>48</sup> R. B. Corey and J. Donohue, *J. Am. Chem. Soc.* 72, 2899 (1950).

<sup>49</sup> D. C. Hodgkin, *Cold Spring Harbor Symposia Quant. Biol.* 14, 65 (1949).



the employment of the intensity of the X-ray reflection in a Fourier expansion series. This allows a map of the electron density in the crystal to be made. Possibly the most impressive work on protein crystals is that of Boyes-Watson, Davidson, and Perutz<sup>50</sup> on methemoglobin crystals containing varying quantities of water.

### THE ELECTRON MICROSCOPE

The resolving power of a microscope is limited by the wavelength of light used, the minimum distance that can be resolved being one-half of the wavelength of light. This means that the smallest particle visible in the very best microscope is about 2000 Å in diameter. Some extension of this resolution is obtained by microscopes using ultra-violet light, and with such microscopes it should be possible to resolve objects that are about 1000 Å in diameter. These microscopes use spherical mirrors instead of lenses. The advantage of mirrors is that the focus of the microscope is independent of wavelength of light. The object under investigation can be located in the microscope field with visible light; photomicrographs using ultra-violet light are then taken without disturbing the setting of the microscope. It is also possible to make quantitative studies of the absorption of ultra-violet light by various portions of the specimen. These can be related to the chemical constitution of various localities in the tissue.

The electron microscope makes use of the same optical principles as does an ordinary microscope except that magnetic or electrostatic fields are used to focus the beam of electrons instead of glass lenses. The specimens show differential transparency to electrons, depending on the electron density of the specimen, and a true picture of the structure is obtained. The electron beam after passing through the specimen is greatly magnified and focused on a photographic plate or on a fluorescent screen. As noted above, the resolving power of a microscope is limited by the wavelength of the light used. The wavelength of an electron beam depends on the potential through which the electrons fall and is in centimeters

$$\lambda = \frac{12.3 \times 10^{-8}}{V^{1/2} + 4.918 \times 10^{-7} V^{3/2}} \quad (15)$$

where  $V$  is the potential drop through which the electron passes expressed in ordinary volts. Most electron microscopes use between 50 and 100 kilovolts, and the wavelength of the electron beam is less than 0.05 Å. For various technical reasons no such resolution is now possible, but under favorable conditions present-day microscopes can resolve objects about

<sup>50</sup> J. Boyes-Watson, E. Davidson, and M. F. Perutz, *Proc. Roy. Soc. (London)* A191, 83 (1947).

20 Å across. Electron microscopes apparently suffer from all the optical troubles of an ordinary microscope plus some of their own. A development that has greatly increased the resolving power of the electron microscope is the shadow technique by means of which a metal such as gold is vaporized on the specimen at an angle to create the appearance of a shadow on one side of the specimen. Owing to the general opaqueness of matter to electrons, the electron beam has to travel in a high vacuum and the specimen likewise is in a vacuum. This means that only desiccated objects can be studied.

Many beautiful electron photomicrographs have been published of numerous objects such as tobacco mosaic virus protein molecules, as well as of many other kinds of virus particles, of the muscle proteins, of collagen fibrils, and of other objects. Wyckoff<sup>51</sup> has written a splendid book dealing with the electron microscope.

### PROBLEMS AND QUESTIONS

1. Distinguish between the modulus of rigidity and the modulus of elasticity.
2. Estimate from Fig. 3b the modulus of elasticity of human hair at 25° C. that is due to the entropy factor alone at the point where the stress due to the entropy is zero. Calculate your answer in terms of the elastic element of hair, remembering that 20 per cent of hair is composed of cuticle and that hair saturated with moisture contains about 40 per cent water. Assume the density of the dry protein to be 1.35.

*Ans.:  $E_s = 7.5 \times 10^8$  dynes per sq. cm.; mol. wt. equals about 135.*

3. Explain why  $\alpha$ -keratin shows long-range elasticity, whereas silk fibroin does not.

<sup>51</sup> R. W. G. Wyckoff, *Electron Microscopy*, Interscience Publishers, Inc., New York, 1949.

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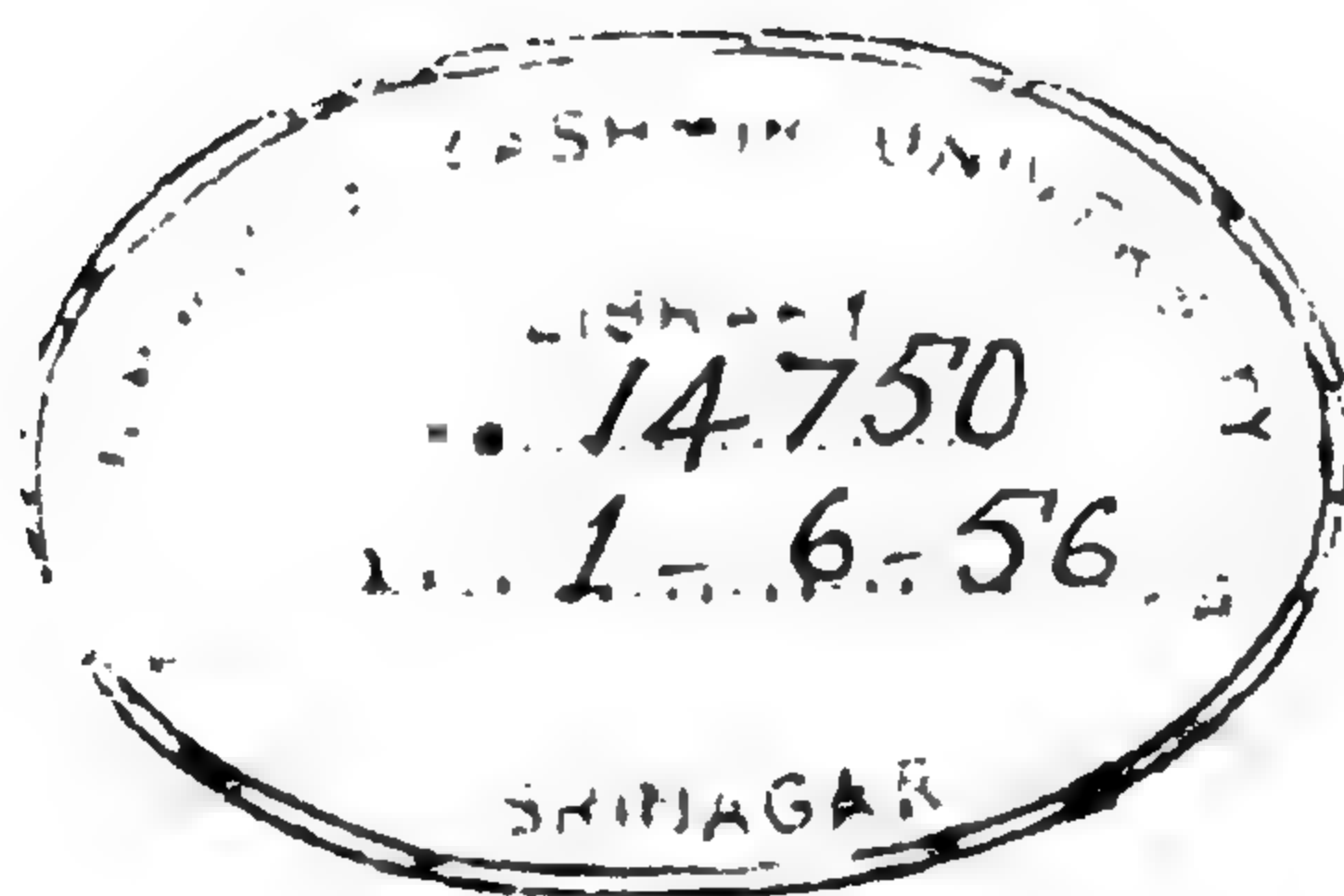
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